CERTIFICATE OF MAILING

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to the: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

TERRI SCHIFFMAN	Terri Schiffman	December 20, 2004
Name	Signature	Date

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Bett, Andrew, et al.	
Serial No.:	09/890,836 - Case No.: 20377YP	Art Unit: 1648
Filed:	August 3, 2001	
For:	IMPROVED HELPER DEPENDENT VECTOR SYSTEM FOR GENE THERAPY	Examiner: Hill, Myron G.

Commissioner for Patents
MAIL STOP APPEAL BRIEF - PATENTS
P. O. Box 1450
Alexandria, Virginia 22313-1450

RESPONSE TO COMMUNICATION PURSUANT TO 37 CFR §1.192(c)

Sir:

This is in response to the outstanding Communication dated December 1, 2004 pursuant to a Notification of Non-compliance with 37 CFR 1.192(c). In that the response is due January 3, 2005, being the first business day after the due date of January 1, 2005, this response is timely and no further extensions of time is needed.

Please substitute the enclosed Amended Appeal Brief Pursuant to 37 C.F.R. §1.192 for the brief that was filed June 28, 2004. As required by 37 CFR §1.192, this brief is being filed in triplicate.

CONDITIONAL PETITION

Applicants hereby make a Conditional Petition for any relief available to correct any defect in connection with this filing, or any defect remaining in this application after this filing. The Commissioner is authorized to charge deposit account 13-2755 for the petition fee and any other fee(s) required to effect this Conditional Petition.

Respectfully submitted,

By _____

Joan E. Switzer
Reg. No. 34,740
Attorney for Applicant

Merck & Co., Inc.
P. O. Box 2000
Rahway, New Jersey 07065-0907

Date: December 20, 2004

AF/1648/#

CERTIFICATE OF MAILING

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Commissioner for Patents
MAIL STOP APPEAL BRIEF – PATENTS
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Alexandria, Virginia 22313-1450

AMENDED APPEAL BRIEF PURSUANT TO 37 C.F.R. §1.192

Sir:

This is an appeal to the Board of Appeals from a decision mailed January 14, 2004 in which the Examiner finally rejected claims 1-15 of the above-identified application. Applicant has timely filed a Notice of Appeal by certification on April 14, 2004. This brief is being filed in support of that Notice of Appeal.

The date of the Notice of Appeal according to the USPTO is April 28, 2004. Therefore, this brief is due June 28, 2004 under 37 C.F.R. §1.136 (a). Therefore, this brief is timely filed.

Kindly charge $320 to Deposit Account No. 13-2755 in the name of Merck and Co., Inc. This amount reflects the filing fee set forth in 37 C.F.R. §1.17 (a)(2). As required by 37 C.F.R. §1.192, this brief is being filed in triplicate. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No.13-2755 in the name of Merck and Co., Inc.

1. REAL PARTY IN INTEREST

The real party in interest in the present appeal is:

Merck and Co., Inc.

One Merck Drive

Whitehouse Station, New Jersey 08889

having acquired the entire right, title and interest for U.S. Patent Application Serial No. 09/890,836 from the Applicants, Andrew Bett, Volker Sandig and Rima Youil, by way of an Assignment.

2. RELATED APPEALS AND INTERFERENCES

No related appeals or interferences are known to appellants or appellants' legal representative which will directly affect or be directly affected by or have bearing on the Board's decision in this appeal.

3. STATUS OF CLAIMS

Claims 1-15 are presently pending in the application. Claims 1-15 stand finally rejected under 35 U.S.C. §112, second paragraph. Claims 1-5 and 7 and 6, 8 and 9-12 stand finally rejected under 35 U.S.C. §102 (b). The rejections of claims 1-15 under §112 and claims 1-5 and 7 and 6, 8 and 9-12 under §102 (b) are being appealed.

4. STATUS OF AMENDMENTS

In an Office Action dated January 14, 2004, the Examiner finally rejected claims 1-15. In response thereto, Applicants subsequently filed an Amendment on April 14, 2004 canceling claim 8, amending claims 1, 6, 7 and 10, and maintaining claims 4 and 14 which had been previously presented. Applicants filed a Supplemental Amendment on May 25, 2004 amending claim 1. The Supplemental Amendment erroneously indicated that claim 10 had been currently amended when it should have been listed as one previously presented. The Supplemental Amendment was filed in conformance with Applicants' legal representative's understanding of the suggestions made by the Examiner during telephonic interviews conducted on May 20 and 21, 2004.

An Advisory Action, dated May 24, 2004, and an Interview Summary, dated May 25, 2004, respectively, were received on May 27, 2004, subsequent to the filing of the Supplemental Amendment summarizing the telephonic interviews conducted on May 20, 2004 and May 21, 2004, respectively. The Advisory Action indicated that the Amendment was not entered in the case.

The claims that were pending as of the Final Office Action are appended as Appendix I. The claims that were presented, but not entered, in the Supplemental Amendment are appended as Appendix II.

For the convenience of the Board of Appeals and Interferences, three copies of the papers listed above are enclosed.

Also enclosed are three copies of the following publications discussed during the prosecution and in this Appeal Brief:

(a) Hardy *et al.*, International Patent Publication WO 97/32481 ("Hardy");

(b) Gräble, M. and Hearing, P., J. Virol., May 1990, Vol. 64, No. 5, p. 2047-2056 ("Gräble and Hearing, 1990");

(c) Gräble, M. and Hearing, P., J. Virol., Feb. 1992, Vol. 66, No. 2, p. 723-731 ("Gräble and Hearing, 1992"); and

(d) Schmid, S. and Hearing, P, J. Virol., May 1997, Vol. 71, No. 5, p. 3375-3384 ("Schmid and Hearing").

5. SUMMARY OF THE INVENTION

The embodiment of the invention that is under consideration in this application is directed to a novel element that has been incorporated into known helper virus vectors. The novel element is an adenovirus packaging signal cassette having low homology to, and less activity than, a corresponding wild-type packaging signal. The modified packaging signal is used in a helper virus to decrease recombination and generation of the virus.

A. Background

Homology between a helper and the helper-dependent adenoviral vector encourages recombination events between the two, resulting in unwanted changes in the structure of the helper-dependent adenoviral vector of the helper virus, and leading to an increased contamination by helper virus. Sequences for different low homology excisable packaging signal cassettes can be designed by one of ordinary skill in the art using the wild-type packaging signal sequences. The wild-type packaging signal of adenovirus serotype 5 is formed by at least seven functional units called A repeats, which are located between nt 230 and nt 380 of the genome. The A elements have the consensus sequence ATTTGN8CG, identified by Schmid and Hearing.

B. Invention

The low homology packaging signal cassette of the instant invention comprises a modified packaging sequence which fulfills the role of an adenovirus packaging signal and has low

homology relative to a corresponding wild-type adenovirus packaging signal. The modified packaging sequence of the instant invention has (1) fewer packaging elements, i.e. less A repeats, than the wild-type adenovirus packaging signal and (2) A elements that have been changed from their corresponding wild type. Specifically, in the claimed invention Applicants have found that a low homology packaging signal could be designed using less than seven A elements. In a preferred embodiment, the modified packaging signal has two to six A elements. The embodiment of the invention further comprises A elements that have been modified relative to the corresponding wild-type sequence in order to reduce contiguous sequence homology. In the modified A element, the eight ambiguous nucleotides (N8) of the consensus sequence within each A element have been replaced by sequences taken from a different A element. By way of example, the eight nucleotides with the first A element ("A1") were replaced by those from the fifth ("AV"), while the eight nucleotides with the second A element ("AII") with the nucleotides from the sixth A element ("AVI"). In addition, a modified A element can be created by changing the corresponding wild-type nucleotides to those of the consensus sequence, such as was done in the instant invention by creating a new A element between the AII and AIII starting 21 base pairs after AII. A modified A element can also be created by changing one or more of the unambiguous nucleotides of the consensus sequence such as was done in AIV; ATTTTGTGTT (SEQ ID NO. 2) was changed to ATTTTGTTGT (SEQ ID No. 3).

The following illustrates how the elements of claims 1-15 as they are presented in the Supplemental Amendment read on the specification.

Claim 1

Claim element	**Where found in the specification**
a nucleic acid molecule comprising a low homology packaging signal cassette	page 3, lines 19-22; page 5, lines 7-9
flanked by a recombinase recognition sequence	page 3, lines 24-27
a modified adenovirus packaging signal	page 3, lines 31-33; page 4, lines 1-7
having one to five A elements	page 11, lines 17-23; Example 2
each A element having a consensus sequence of ATTTGN8CG (SEQ ID NO. 1)	page 11, lines 15-17; Example 2
where N8 of each A element is replaced by the N8 sequence of a different A element	page 11, lines 25-34; Example 2
all N8 sequences are not identical	page 11, lines 25-34; Example 2

Claim 6

Claim element	Where found in the specification
maximum, 23 bp of contiguous sequence homology	page 4, lines 1-7
relative to a wild-type packaging signal	page 4, lines 1-7
at a portion of the sequence other than the A elements	page 4, lines 1-7; Example 2

Claim 7

Claim element	Where found in the specification
2-3 times less efficient than said wild-type signal	page 4, lines 8-15
relative to adenovirus within the same cell line	Page 4, lines 8-15

6. ISSSUES

There are three issues on appeal:

(A) whether claims 1-15 are indefinite under 35 U.S.C. §112, second paragraph;

(B) whether claims 1-5 and 7 are anticipated under 35 U.S.C. §102(b) by Hardy et al., WO 97/032481; and

(C) whether claims 6, 8 and 9-12 are anticipated under 35 U.S.C. §102(b) by Hardy et al., WO 97/032481.

7. GROUPING OF CLAIMS

With respect to the rejection under 35 U.S.C. §112, second paragraph, all the claims stand or fall together.

With respect to the rejection under 35 U.S.C. §102 (a) all the claims stand or fall together.

With respect to the rejection under 35 U.S.C. §102 (b) all the claims stand or fall together.

8. ARGUMENT

A. Summary of the Examiner's Position

The Appellants believe that the Examiner's reason for holding that claims 1-15 are indefinite can be summarized as follows:

Since the claims are directed to a low homology packaging signal, the claims must contain a function or property of the packaging signal that explicitly confers low homology to the construct relative to a wild-type signal.

Since claim 7 is directed to a modified packaging signal that is 2-3 times less efficient it must be definite as to how this efficiency is to be measured relative to a wild-type signal.

Since claim 8 is directed to an A element, it must be definite as to what is the consensus sequence.

Since claim 10 is directed to a helper virus it must be definite as to how the virus can be the nucleic acid of claim 6.

The Appellants believe that the Examiner's reasons for holding that claims 1-5 and 7 and 6, 8 and 9-12 are anticipated can be summarized as follows:

Hardy *et al.*, WO 97/32481 ("Hardy") disclosed the use of a low homology packaging signal containing A elements. Applicants use of the term "low homology" is insufficient to distinguish the claimed construct from that disclosed by Hardy. Applicants use of additional features in dependent claims is insufficient to distinguish the claimed invention from Hardy in that the claim from which they depend from is not novel.

A. Summary of the Appellants Position

With respect to the claims as they appear in the Supplemental Amendment and the indefiniteness rejection:

Applicants have amended claim 1 to incorporate the structure of previous claim 8 to further define the low homology packaging signal as suggested by the Examiner in a telephone interview. Applicants have amended claim 1 to further define the modified packaging signal as having one to five A elements, each A element having a consensus sequence (ATTTGN8CG, SEQ ID NO. 1), with N8 being replaced by the N8 sequence of a different A element and all A elements are not identical.

Applicants have amended claim 7 to further define and distinguish the claimed invention by specifying the location of the 23 bp of contiguous sequence homology.

Applicants have amended claim 10 to further define and distinguish the claimed invention by specifying that a helper virus comprises the nucleic acid of claim 6.

With respect to the claims as they appear in the Supplemental Amendment and the anticipation rejections:

Hardy et al. does not teach all of the elements of the modified packaging signal that comprises the low homology packaging signal claimed by the Applicants. Hardy does not teach any A element having a modified consensus sequence. Hardy does not teach the use of 23 bp of contiguous sequence homology at a portion of the sequence other than the A elements. Hardy does not teach a packaging signal that is less efficient relative to an adenovirus within the same cell line.

Claims 1-15 are patentable under 35 U.S.C. §112, second paragraph, as they clearly convey and distinctly claim the subject matter claimed therein.

Claims 1-15 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. In particular, the above claims were alleged to be indefinite as to the metes and bounds of what a "low homology" packaging signal is relative to a wild-type signal. Claim 7 was rejected as it was alleged that it was not clear to what the phrase "2-3 times less efficient" relates. Claim 8 was rejected for alleged lack of clarity as to what is the consensus sequence. Claim 10 was rejected for alleged lack of clarity as to how a nucleic acid can be a helper virus. Applicants respectfully traverse these rejections and submit that they are not sufficient.

In the Supplemental Response, Applicants have amended claim 1 to more distinctly define and distinguish the invention by adding the feature that the packaging signal has one to five A elements and that each A element has a consensus sequence, ATTTGN8CG (SEQ ID NO. 1), which features were previously in dependent claim 8. Claim 1 has been further amended to specify that the ambiguous N8 sequence of each A element is replaced by the N8 sequence of a different A element and that all N8 sequences are not identical.

Similarly, Applicants have amended claim 7 to further recite that the decreased efficiency of the modified packaging signal of claim 5 (which depends ultimately from claim 1) relative to a wild-type packaging signal is relative to an adenovirus within the same cell line. While Hardy may suggest that constructs in different host cells have different packaging (page 39, line 18-20), a full reading of that paragraph makes it evident that the comparison the reference is making is across cell lines (page 39, lines 13-20). Applicants have defined "less efficient" on page 4, lines 8-15 of the Specification, and have

defined that the claimed term refers to a comparison of the modified packaging signal relative to a wild-type adenovirus packaging signal within the same cell line, not across different types of cell lines.

The embodiment of the invention set forth in claim 8 has been incorporated into claim 1and claim 8 has been canceled, rendering this rejection moot.

Applicants have amended claim 10 in the Supplemental Response to clarify that the claimed helper virus comprises the nucleic acid molecule of claim 6.

Applicants assert that, based on the amendments made and the information provided within the Specification, one of ordinary skill in the art would be able to appreciate the nature of the low homology, modified adenovirus packaging signal claimed and to further find and identify the species encompassed. This alone supports the mandates of 35 U.S.C. §112 which requires only that the scope of the claim be "clear to a hypothetical person possessing the ordinary level of skill in the pertinent art," see MPEP §2171.

In evaluating claims with regard to Section 112, "definiteness of claim language must be analyzed, not in a vacuum, but in light of : (a) the content of the particular application disclosure; (b) the teachings of the prior art; and (c) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made," see MPEP §2173.02. In this regard, Applicants point out that the Specification, the application disclosures which details, *inter alia*, all of the structural and functional characteristics discussed above, describes the A elements and the changes made to the A elements in detail more than sufficient to enable "one possessing the ordinary level of skill in the pertinent art" to ascertain the scope of the patent claims.

The claim terminology "low homology" as used relevant to a modified adenovirus packaging signal is not a means for rejecting the claims, as the term itself is very specific when used in the context of adenovirus packaging signals. Proper enablement does not equate with importing all characteristics of the packaging signal detailed in the Specification into the claims. As indicated above, "whether a claim is invalid for indefiniteness requires a determination whether those skilled in the art would understand what is claimed when the claim is read in light of the specification," Morton International, Inc. v. Cardinal Chemical Co., 28 USPQ 2d 1190, 1194 (Fed. Cir. 1993), on remand from, 26 USPQ 2d 1721 (1993).

The claimed packaging signal molecules are thoroughly described and detailed in the Specification and the terminology used is certainly sufficient to put one of ordinary skill in the art on notice of the scope of the claims. Further, the claims incorporate sufficiently descriptive elements with antecedent support in the Specification in order to clearly and distinctly convey exactly what the invention entails.

Applicants, therefore, submit that the instant claims are not indefinite, but rather sufficiently descriptive to very clearly and distinctly convey the scope of the invention.

Applicants, therefore, respectfully request that this objection be overturned.

Claims 1-5 and 7 are not anticipated by Hardy.

The above claims are rejected based on Hardy which discloses methods for producing, *in vivo*, helper-free, totally defective adenovirus vectors for use in gene therapy. The recombinant adenovirus vectors described therein are packaged using a helper virus which can be excised *in vivo* by recombination mediated by a recombinase. It has been asserted that Hardy teaches a packaging signal construct having low homology, i.e. a nucleic acid molecule for use as a helper virus that is inefficiently packaged (as taught on page 39). Applicants submit that in light of the claim amendments presented in the Supplemental Response, this is not a sufficient rejection.

As noted above, in the Supplemental Response Applicants amended claim 1, and in turn the claims depended therefrom, to include additional features, namely, the number and type of A elements. More specifically, claim 1 states that the modified packaging signal has one to five A elements, which is less than the seven A elements of the wild-type packaging signal, and wherein each A element has a consensus sequence, ATTTGN8CG (SEQ ID NO. 1). Further, it has been specified that the ambiguous N8 sequence of each A element is replaced by the N8 sequence of a different A element, such that all N8 sequences differ from their corresponding wild-type A element and all are not identical.

Hardy defines that the packaging signal used therein contains an adenovirus packaging site *in cis* for packaging of the DNA into the adenovirus vectors (page 16, lines 1-2). The packaging sites exemplified in Hardy are directed to mutations of the naturally occurring adenovirus packaging sites. Hardy states that, in addition to the naturally occurring adenovirus packaging sites, certain other DNA sequences have been shown empirically to function as packaging sites, i.e. synthetic packaging sites. Id. at lines 4-9. The only example given in Hardy of such a synthetic packaging site *in vivo* is given as a cross reference to Gräble and Hearing, 1990, in which one synthetic packaging site composed of six tandemly repeated copies of the A repeat was used as a packaging site. Id. at lines 15-19. A further review of the A repeats of Gräble and Hearing, 1990, makes it apparent that they utilized an A repeat (A/T-AN-A/T-TTTG) with a consensus sequence that differed from that claimed by the Applicants.[1]

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[1] This consensus sequence was later modified by Gräble and Hearing, 1992 (GTN3-4TTTG), and was modified yet again in Schmid and Hearing to the presently accepted A element consensus sequence (ATTTGN8CG).

Thus, one of ordinary skill in the art would find no teaching either explicitly or inherently of the modified A elements claimed by the Applicants.

The Examiner has suggested in the May 25, 2004 Communication, received after the filing by Applicants' legal representative of the Supplemental Response, that Applicants should not claim a construct comprising one or two A elements, presumably inferring from Gräble and Hearing, 1990, that such a construct would not be functional. However, because Gräble and Hearing, 1990 used identical sequences (which were not the consensus sequences of the instant invention) in tandem as A elements and did not use a modified packaging signal comprising non-identical modified A elements (with the altered N8 sequences) which were not in tandem, one of ordinary skill in the art would not infer the teachings as to functionality of these constructs to the instant invention. On the contrary, upon a further review of the third construct in both Fig. 3 and Fig. 7 one skilled in the art may well infer that a construct which has two A elements that are not in tandem may function as a packaging signal *in vivo*.

Applicants submit that, absent any disclosure in either Hardy or Gräble and Hearing, 1990 as to the use of a modified packaging signal comprising the modified A elements, there can be no anticipation of claim 1 and the claims that depend therefrom. A valid anticipation requires "disclosure in [a] single prior art reference of each element of [the] claim under consideration," W.L. Gore & Assoc., Inc. v. Garlock, Inc., 721 F.2d 1540, 1554, 220 USPQ 303, 313 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984).

Applicants, therefore, respectfully request that this rejection be overturned.

Claims 6, 8 and 9-12 are not anticipated by Hardy.

The above claims are rejected based on Hardy which discloses methods for producing, *in vivo*, helper-free, totally defective adenovirus vectors for use in gene therapy. As stated above, the recombinant adenovirus vectors described therein are packaged using a helper virus which can be excised *in vivo* by recombination mediated by a recombinase. It has been asserted that Hardy teaches a heterologous packaging signal construct that is equivalent but not identical and not co-linear as indicated by genome position. It is further asserted that these constructs can be plasmids and can contain deletion of E1 and be a helper virus. Applicants submit that in light of the claim amendments presented in the Supplemental Response, this is not a sufficient rejection.

As noted above, the Supplemental Response amended Claim 6 to further specify that the 23 bp of contiguous sequence homology is relative to a wild-type packaging signal at a portion of the sequence other than the A elements. Claim 6 depends ultimately from claim 1 and thus incorporates the structural features included thereof. Applicants do not refute the assertions (set forth in an earlier Office

Action, but still maintained in the Final Office Action) that Hardy discloses the use of Ad7 packaging signals, constructs which can be plasmids, which can contain deletion of the E1 region or be a helper virus and/or which may accommodate inserts of about 2.9 Kb. However, neither Hardy nor Gräble and Hearing, 1990, cited therein, disclose a construct and/or a helper virus that uses these features in combination with the structural features of claim 1. Further, there is no disclosure in Hardy of the use of a further feature of the claimed construct of a maximum of 23 bp of contiguous sequence homology relative to a corresponding wild-type signal at a portion of the sequence other than the A elements.

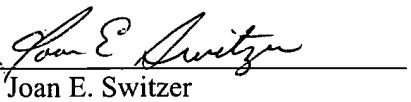
Applicants submit that, absent any disclosure in either Hardy or Gräble and Hearing, 1990 as to the use of these elements in addition to the structural features of claim 1, there can be no anticipation of claims 6 and 9-12. A valid anticipation requires "disclosure in [a] single prior art reference of each element of [the] claim under consideration," W.L. Gore & Assoc., Inc. v. Garlock, Inc., 721 F.2d 1540, 1554, 220 USPQ 303, 313 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984).

Applicants, therefore, respectfully request that this rejection be overturned.

CONCLUSION

In view of the above, the Appellants respectfully submit that it has been demonstrated that claims 1-7 and 9-15 are definite and are not anticipated by the cited prior art. Therefore, the Appellants request that the Board of Patent Appeals and Interferences reverse the outstanding rejections of claims 1-7 and 9-15 under 35 U.S.C. §112 and 35 U.S.C. §102 (b).

Respectfully submitted,

By 

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Date: December 20, 2004

APPENDIX I

1. (original) A nucleic acid molecule comprising a low homology packaging signal cassette flanked by a recombinase recognition sequence, wherein said packaging signal cassette comprises a modified adenovirus packaging signal, provided that said modified packaging signal has low homology to a wild-type adenovirus packaging signal.

2. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *loxP*.

3. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *frt*.

4. (previously presented) The nucleic acid of claim 1, wherein said modified packaging signal is less efficient than said wild-type packaging signal.

5. (original) The nucleic acid of claim 4, wherein said wild-type packaging signal is human adenovirus serotype 5 packaging signal.

6. (currently amended) The nucleic acid of claim[[s]] 5, wherein the modified packaging signal comprises at a maximum, 23 bp of contiguous sequence homology with said wild-type packaging signal.

7. (original) The nucleic acid of claim 5, wherein said modified packaging signal is about 2-3 times less efficient than said wild-type signal.

8. (currently amended) The nucleic acid of claim 6, wherein said modified packaging signal comprises two to six A elements, each A element having a consensus sequence of ATTTGN8GC (SEQ ID NO: 1).

9. (original) The nucleic acid of claim 6, wherein said nucleic acid is a plasmid.

10. (currently amended) The nucleic acid of claim 6, wherein said nucleic acid [[is]] comprises a helper virus.

11. (original) The nucleic acid of claim 10, wherein said helper virus does not contain an E1 gene.

12. (original) The nucleic acid of claim 11, wherein said helper virus comprises an E3 region with an insert of about 2.9 kb.

13. (original) The nucleic acid of claim 12, wherein said insert does not contain a promoter sequence.

14. (currently amended) A nucleic acid of claim 13 comprising an adenovirus E3 gene having an insertion of at least about 2.7 kb, provided that said insertion does not contain a promoter sequence.

15. (original) The nucleic acid of claim 14, wherein said insertion is a human intron sequence.

16 - 41. (canceled)

APPENDIX II

1. (currently amended) A nucleic acid molecule comprising a low homology packaging signal cassette flanked by a recombinase recognition sequence, wherein said packaging signal cassette comprises a modified adenovirus packaging signal having one to five A elements, each A element having a consensus sequence of ATTTGN8CG (SEQ ID NO:1), and where N8 of each A element is replaced by the N8 sequence of a different A element and all N8 sequences are not identical.

2. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *loxP*.

3. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *frt*.

4. (previously presented) The nucleic acid of claim 1, wherein said modified packaging signal is less efficient than said wild-type packaging signal.

5. (original) The nucleic acid of claim 4, wherein said wild-type packaging signal is human adenovirus serotype 5 packaging signal.

6. (previously presented) The nucleic acid of claim 5, wherein the modified packaging signal comprises at a maximum 23 bp of contiguous sequence homology relative to a wild-type packaging signal at a portion of the sequence other than the A elements.

7. (previously presented) The nucleic acid of claim 5, wherein said modified packaging signal is about 2-3 times less efficient than said wild-type signal relative to adenovirus within the same cell line.

8. (cancelled)

9. (original) The nucleic acid of claim 6, wherein said nucleic acid is a plasmid.

10. (currently amended) A helper virus comprising the nucleic acid of claim 6.

11. (original) The nucleic acid of claim 10, wherein said helper virus does not contain an E1 gene.

12. (original) The nucleic acid of claim 11, wherein said helper virus comprises an E3 region with an insert of about 2.9 kb.

13. (previously presented) The nucleic acid of claim 12, wherein said insert does not contain a promoter sequence.

14. (original) A nucleic acid of claim 13 comprising an adenovirus E3 gene having an insertion of at least about 2.7 kb, provided that said insertion does not contain a promoter sequence.

15. (original) The nucleic acid of claim 14, wherein said insertion is a human intron sequence.

HELPER-FREE, TOTALLY DEFECTIVE ADENOVIRUS FOR GENE THERAPY

ACKNOWLEDGEMENTS

This invention was made with Government support under Grants Nos. P01-NS16033 and R01-NS13521, awarded by the National Institutes of Health. The U.S. Government has certain rights in this invention.

INTRODUCTION

Background

Adenoviruses (Ads) belong to the family *Adenoviridae* and the human Ads belong to the genera *Mastadenovirus*. Human Ad infections are found worldwide. Ads were initially characterized in 1953 by Rowe *et al.* when trying to cultivate epithelial cells from the adenoids. The 47 different serotypes are grouped (A-F) according to their ability to cause tumours in newborn hamsters. Respiratory epithelial cells are the primary target for Ads *in vivo*. 5% of the acute respiratory diseases in children under the age of 5 are due to Ads. Other sites of infection include the eye, the gastro-intestinal tract and the urinary tract. Many Ad infections are subclinical and only result in antibody formation.

Three loosely defined sets of protein exist in the mature Ad: proteins that form the outer coat of the capsid, scaffolding proteins that hold the capsid together and DNA-binding proteins. The diameter of the icosahedral-shaped capsid varies from 65 to 80 nm depending on the serotype. The capsid is composed of a total of 720 hexon and 60 penton subunit proteins, 360 monomers of polypeptide VI, 240 monomers of polypeptide IX, and 60 trimeric fibre proteins.

Bound to the penton subunits and protruding from the capsid is the fibre protein which mediates the initial attachment of the virus to a target cell. Polypeptides IX, IIIa, and VI form the scaffolding which holds the capsid together. Polypeptide IX stabilizes the packing of adjacent hexons in the capsid, polypeptide IIIa spans the capsid to link hexons of adjacent faces, and polypeptide

VI connects the structural proteins to the core. The core consists of DNA associated with polypeptides V, VII, μ and the terminal protein.

Ads contain double stranded DNA as their genetic material. The base composition of the 47 characterized serotypes (Ad1-Ad47) varies in the percent G + C content and in the length of the genome (approximately 36 kb) and of the inverted terminal repeats (100-140 bp). The genome is covalently linked at each 5′ end to individual 55 kd terminal proteins, which associate with each other to circularize the DNA upon lysis of the virion.

The Ad genome is functionally divided into 2 major non-contiguous overlapping regions, early and late, based on the time of transcription after infection. The early regions are defined as those that are transcribed before the onset of viral DNA synthesis. The switch from early to late gene expression takes place about 7 hours after infection. The terms early and late are not to be taken too literally as some early regions are still transcribed after DNA synthesis has begun.

There are 6 distinct early regions; E1a, E1b, E2a, E2b, E3, and E4, each (except for the E2a-b region) with individual promoters, and one late region, which is under the control of the major late promoter, with 5 well characterized coding units (L1-L5). There are also other minor intermediate and/or late transcriptional regions that are less well characterized, including the region encoding the viral-associated (*VA*) RNAs. Each early and late region appears to contain a cassette of genes coding for polypeptides with related functions. Each region is transcribed initially as a single RNA which is then spliced into the mature mRNAs. More than 30 different mature RNA transcripts have been identified in Ad2, one of the most studied serotypes.

Once the viral DNA is inside the nucleus, transcription is initiated from the viral E1a promoter. This is the only viral region that must be transcribed without the aid of viral-encoded *trans*-activators. There are other regions that are also transcribed immediately after cell infection but to a lesser extent, suggesting that the E1 region is not the only region capable of being transcribed without viral-encoded transcription factors. The E1a region codes for more than six polypeptides. One of the polypeptides from this region, a 51 kd protein,

transactivates transcription of the other early regions and amplifies viral gene expression. The E1b region codes for three polypeptides. The large E1b protein (55 kd), in association with the E4 34 kd protein, forms a nuclear complex and quickly halts cellular protein synthesis during lytic infections. This 55 kd polypeptide also interacts with p53 and directly inhibits its function. A 19 kd *trans*-activating protein encoded by the E1B region is essential to transform primary cultures. The oncogenicity of Ads in new-born rodents requires the E1 region. Similarly, when the E1 region is transfected into primary cell cultures, cell transformation occurs. Only the E1a region gene product is needed to immortalize cell cultures.

The E2a and E2b regions code for proteins directly involved in replication, i.e., the viral DNA polymerase, the pre-terminal protein and DNA binding proteins. In the E3 region, the 9 predicted proteins are not required for Ad replication in cultured cells. Of the 6 identified proteins, 4 partially characterized ones are involved in counteracting the immune system; a 19 kd glycoprotein, gp19k, prevents cytolysis by cytotoxic T lymphocytes (CTL); and a 14.7 kd and a 10.4 kd/14.5 kd complex prevent, by different methods, E1a induced tumour necrosis factor cytolysis. The E4 region appears to contain a cassette of genes whose products act to shutdown endogenous host gene expression and upregulate transcription from the E2 and late regions. Once viral DNA synthesis begins, the late genes, coding mainly for proteins involved in the structure and assembly of the virus particle, are expressed.

Recombinant human adenoviruses have attracted much attention of late because of their potential for gene therapy and gene transfer and for protein expression in mammalian cells. First-generation recombinant adenovirus vectors most often contain deletions in the E1a and/or E1b regions. The usefulness of such vectors for gene transfer has been demonstrated in mice, cotton rats and nonhuman primates (Engelhardt *et al.* Hum. Gene Ther. 4:759-769 1993; Rosenfeld *et al.* Cell 68:143-155 1992; Yang *et al.* Nat. Genet. 7:362-369 1994). A fundamental problem encountered in using these vectors for gene therapy, however, is that deletion of the E1 sequences alone is not sufficient to completely ablate expression of other early and late viral genes or to prevent replication of the

viral DNA. Studies have indicated that these vectors express viral antigens which elicit destructive immune responses in the target cells (Yang *et al.* Proc. Natl Acad. Sci.91:4407-4411 1994; Yang *et al.* Nat. Genet. 7:362-369; Yang *et al.* J. Virol. 69:2004-2015 1995). This immune response leads to loss of transgene expression and development of inflammation. In addition, there is indication that memory-type immune responses may substantially diminish the efficiency of gene transfer following a second and subsequent administrations of the recombinant vector (Kozarsky *et al.* J. Biol. Chem. 269:1-8 1994; Smith *et al.* Nat. Genet. 5:397-402 1993). Newer recombinant adenovirus vectors contain additional disabling mutations in other regions of the adenovirus genome, for example in E2a (Englehardt *et al.* Hum. Gene Ther. 5:1217-1229 1994; Englehardt *et al.* Proc. Natl Acad. Sci. 91:6196-6200) or E3 (Bett *et al.* Proc. Natl Acad. Sci. 91:8802-8806 1994). These vectors, although they express fewer viral proteins, do not completely eliminate adenoviral protein expression and so are subject to similar immune response problems as found with the earlier vectors.

In addition to the immune response problems associated with the use of the current adenovirus-based gene therapy vectors, only relatively small amounts of foreign DNA (that is, non-adenovirus DNA) can be accommodated in these vectors due to the size constraints of adenoviral packaging. Studies have shown that adenovirus virions can package up to approximately 105% of the wild type adenovirus genome length (the wild type adenovirus genome is between 35-36 kilobases). Recombinant vectors having deletions in the E1 region typically permit the insertion of less than 5 kb of foreign DNA. Recombinant vectors having additional deletions in E3 can accommodate inserts of up to about 8 kb.

Another serious problem inherent in the use of current recombinant adenovirus-based vectors is their ability to recombine with adenoviruses from natural sources to produce infections of wild type viruses.

It would be advantageous to develop a recombinant adenovirus vector that is incapable of producing any adenovirus proteins, that can accommodate large inserts of foreign DNA and that recombines only at low frequency or not at all with other adenoviruses. The present inventor has surprisingly found that recombinant adenovirus (rAd) vectors containing as little as 600 base pairs of

adenovirus sequence can be replicated and packaged *in vivo* to produce infectious virions. Adenoviral factors necessary for the replication and packaging of the minimal rAd vectors are supplied *in trans* from a recombinant adenovirus helper vector of the present invention which is designed such that the packaging site is easily excisable *in vivo* by the use of the Cre/*lox* recombination system.

Cre/*lox* is a site-specific recombination system, originally discovered in bacteriophage P1, which consists of a recombinase protein (Cre) and the DNA recognition site of the recombinase (Hoess and Abremski in "Nucleic Acids and Molecular Biology", Eckstein and Lilley, eds., Vol. 4, p. 99 Springer-Verlag 1990). Cre (causes recombination) is a member of the Int family of recombinases (Argos *et al.* EMBO J. 5:433 1986) and has been shown to perform efficient recombination of *lox* sites (locus of X-ing over) not only in bacteria but also in eukaryotic cells (Sauer Mol. Cell. Biol. 7:2087 1987; Sauer and Henderson Proc. Natl Acad. Sci. 85:5166 1988). The Cre recombinase can efficiently excise DNA bracketed by *lox* sites from the chromosome. Two components are required for recombination: the Cre recombinase and an appropriate *lox*-containing substrate DNA. Several different *lox* sites have been identified to date, for example *lox* P, *lox* 511, *lox* 514 and *lox* Psym (Hoess *et al.* Nucl. Acids Res. 14:2287-2301 1986). The sequences of the various *lox* sites are similar in that they all contain the identical 13-base pair inverted repeats flanking an 8-base pair asymmetric core region in which the recombination occurs. It is the asymmetric core region that is responsible for the directionality of the site and for the variation among the different *lox* sites. Only *lox* sites having the same sequence are recombined by Cre. Recombination between two directly oriented *lox* sites results in excision of the intervening DNA as a circular molecule having a single *lox* site and leaves a single *lox* site at the point of excision. The intramolecular excision is in equilibrium with the reverse reaction, that is, with intermolecular insertion of a DNA molecule containing a *lox* site into the identical *lox* site remaining in the chromosome. The excision reaction is favored 20 to 1 over the insertion reaction. Recombination between two inversely oriented *lox* sites results in inversion rather than excision of the intervening DNA. Cre/*lox* has been used to remove unwanted DNA sequences from the genome (for example, selectable marker genes when no

longer needed for selection), for designing recombination dependent switches to control gene expression (Sauer and Henderson Nucl. Acids Res. 17:147 1989) and to direct site-specific integration of *lox* vectors into a *lox* site previously placed into the chromosome (Sauer and Henderson New Biol. 2:441 1990).

Relevant Literature

Early experiments showed that it was possible to create defective adenoviruses which carried substitutions of all or part of the SV40 genome in tandem. The deletions included 16% to 29%, 29% to 75% and 75% to 96%, indicating that virtually all of the Ad virus could be substituted. (For a summary of these experiments see, The Adenoviruses, Harold S. Ginsberg, ed. Plenum Press, NY, 1984.)

Bett *et al.* have described an adenovirus vector containing deletions in both the E1 and E3 regions (Proc. Natl Acad. Sci. 91: 8802-8806 (1994)). Mitani *et al.* (Proc. Natl Acad. Sci. 92: 3854-3858 (1995)) have described a recombinant adenoviral vector which is deficient in E1 and contains a 7.23 kb deletion in an essential part of the viral genome carrying L1, L2, VA and TP. A marker gene was inserted in place of the deleted adenoviral DNA and the vector was replicated and packaged after tranfection of 293 cells using a wild type Ad2 virus as a helper. The helper virus was also replicated and packaged. The packaged viruses (wild type helper virus and recombinant virus) were partially separated by repeated CsCl gradient centrifugation.

Anton and Graham (J. Virol. 69: 4600-4606 (1995)) have used Cre-mediated recombination of flanking *lox* P sites to turn on expression of a luciferase gene cloned into an adenoviral vector. The recombination of the *lox* sites resulted in the removal of a fragment of DNA between the luciferase coding sequence and the promoter. The Cre recombinase was supplied from a second adenoviral vector carrying the Cre gene under control of hCMV promoter.

U. S. Patent Number 4,959,317 describes a method for producing site-specific recombination of DNA in eukaryotic cells using Cre-mediated recombination of *lox* sites. Cre-expressing eukaryotic cells are also disclosed. WO 91/09957 describes a method for producing site-specific recombination in

plant cells using Cre-mediated recombination of *lox* sites. EP 0 300 422 describes a method for preparing recombinant animal viral vectors using Cre-mediated recombination between a *lox* P site on the virus and a *lox* P site on a plasmid.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide therapeutic recombinant adenovirus-based (therapeutic rAd) vectors for gene therapy or for expression of foreign genes in mammalian cells. The therapeutic rAd vectors of the present invention contain a minimal amount of adenovirus DNA and are incapable of expressing any adenovirus antigens, i.e. "gutless". The therapeutic rAd vectors of the present invention provide the significant advantage of accommodating large inserts of foreign DNA while completely eliminating the problem of expressing adenoviral genes that result in an immunological response to viral proteins when a therapeutic rAd vector is used in gene therapy. In particular, the therapeutic rAd vector of the present invention comprises the adenovirus inverted terminal repeats, an adenovirus packaging site, one or more *lox* sites and up to 36-38 kb of foreign DNA. By "foreign" DNA is meant any genes or other DNA sequences that do not occur naturally in adenovirus.

The ability of these therapeutic rAd vectors to accommodate such large inserts of foreign DNA (up to 38 kb) permits construction of gene therapy vectors that contain and express extremely large individual genes or polynucleotide sequences as well as multiple genes or polynucleotide sequences. The foreign DNA that can be expressed can be any polynucleotide sequences that do not occur naturally in adenovirus, including the Duchenne Muscular Dystrophy (DMD) gene, all genes involved in dopamine synthesis (e.g. tyrosine hydroxylase, GPD cyclohydroxylase), Factor VIII, Factor IX, superoxide dismutase, GM-CSF (granulocyte-macrophage colony-stimulating factor), genes involved in chronic granulomatous disease (CGD), and multiple genes, including GM-CSF in combination with other cytokines (e.g. interferons (IFN-α, IFN-β, IFN-γ), interleukins, M-CSF (macrophage colony-stimulating factor), tumor necrosis factors, growth factors (TGF-β (transforming growth factor-β) and PDGF (platelet-derived growth factor)), and including GM-CSF with MHC (major

histocompatibility complex) genes. The ability to deliver expression products from extremely large sequences or multiple sequences provides a simple and efficient delivery system.

The present invention includes the discovery that the minimum size range for the rAd vectors is from 32 kb to 38 kb, as smaller rAd vectors (<32 kb) are unstable and not efficiently packaged. This discovery of the lower size limit for packaging efficiency permits increased stability, which is important for vectors intended for gene therapy and increased production efficiency, which can reduce manufacturing costs significantly. Thus, provided herein are methods of producing a rAd vector for gene therapy, comprising constructing a therapeutic rAd vector wherein said vector ranges in total size from 32 kb to 38 kb. The vectors of the invention include both plasmids as well as packaged recombinant viral and foreign DNA.

Because the therapeutic rAd vectors of the present invention do not express any adenovirus proteins, those adenovirus proteins that are required for the replication and packaging of the therapeutic rAd vectors of the present invention are supplied *in trans* by a helper recombinant adenovirus vector (helper rAd).

It is another object of the present invention to provide a helper recombinant adenovirus vector which is useful for the preparation of *in vivo* packaged therapeutic rAd vectors. The helper rAd vector of the present invention comprises adenovirus genes which encode proteins necessary for the replication and packaging of the therapeutic rAd vectors into therapeutic rAd virus particles. The helper rAd vector of the present invention additionally comprises an adenovirus packaging site flanked by at least one set of two identical *lox* sites in direct orientation. When the helper rAd of the present invention is grown in a host cell that produces Cre recombinase, the packaging site is excised by Cre-mediated recombination between the flanking *lox* sites. Since the presence of an adenovirus packaging site is absolutely required for packaging of the DNA into adenovirus virions, removal of the packaging site by excision prevents the helper rAd vector from being packaged. One of ordinary skill in the art will understand that the host cells useful in the invention can be any cell lines susceptible to adenovirus infection and capable of expressing a recombinase capable of mediating

recombination between recombination sites. Any recombinase-expressing cell line and its corresponding recombination sites can be used, including, but not limited to, the FLP recombinase and its recombination site. See O'Gorman et al., Science 251:1351 (1991). The term recombinase includes any enzymes that mediate recombination between its corresponding recombination sites, which are nucleic acid sequences that are specifically recognized by the recombinase. One of ordinary skill in the art will readily appreciate that any examples describing cre-recombinase and lox sites can be substituted with any other recombinase and its corresponding recombination sites.

Generally, the helper rAd vector of the present invention comprises all of the adenovirus genes necessary to provide the replication and packaging functions but may contain less than all of the necessary genes if the proteins encoded by some of these adenovirus genes are supplied in other ways, for example, by the host cell. In particular, the helper rAd vector need not contain the adenovirus E1a and E1b regions if used in combination with a host cell that can supply E1a and E1b gene products. If such a cell line is used, the E1a gene promoter should be transcribed from a heterologous promoter. It is essential that the Ad packaging site in the E1a promoter enhancer is not present in the host cell line.

It is a further object of the present invention to provide a system and a method for preparing a substantially pure preparation of *in vivo* packaged therapeutic rAd vector particles. The method of the present invention comprises transfecting an appropriate host cell with therapeutic rAd vector DNA and helper rAd vector DNA. The transfected cells are cultured for a sufficient time to allow maximum production of the therapeutic rAd virus. The virus particles are harvested and used to infect fresh host cells either with or without the addition of a small amount of packaged helper rAd virus particles. The virus particles produced following infection are isolated and the infection process may be repeated. In the final step, Cre-expressing host cells are infected with the viral particles produced in the earlier infections. Infection of the Cre-expressing cells provides for selection against the helper rAd so that a substantially pure preparation of packaged therapeutic rAd vector particles is produced.

Cre-expressing mammalian host cell cultures are also provided.

Also provided are methods and systems for rapidly and efficiently generating new recombinant adenovirus vectors with substitutions in a adenoviral region. These methods and systems comprise a helper rAd vector, a replicating vector containing ITRs, and Ad packaging site, substitute DNA and a recombination site identical to at least one of the recombination sites in the helper rAd vector, and a recombinase-expressing host cell line. One of skill in the art will appreciate that the vectors can be either recombinant adenoviruses or plasmids. These methods and systems provide a simple and efficient alternative to existing overlap recombination techniques. A working stock of virus can be produced for initial experiments within 10 days. Moreover, substituted rAds can be generated in substantially pure form without need for plaque purification. This solves the problem of purifying the recombinant adenovirus vectors with substitutions away from the rAd helper vectors. Other advantages of these methods are that (a) viral sequences in plasmids are more stable and easier to prepare than viral DNA, which is under continuous selection during growth and (b) recombinase-mediated recombination enables use of a small replicating vector which is extremely easy to manipulate. One of ordinary skill in the art will appreciate that both replicating and nonreplicating vectors can be used in these methods and systems.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. The genetic map of a wild type Adenovirus.

FIG. 2. Nucleotide sequence of several different *lox* sites.

FIG. 3. Schematic representation of the helper rAd helper vector Ψ5. The bottom figure represents the complete Ψ5 genome. The upper figure shows the details of the structure of the insert carrying the packaging site flanked by the lox P sites which is substituted into the Ad5 at position 0-3328.

ITR = inverted terminal repeat; P_{CMV} = CMV promoter; P_{E1a} = E1a promoter; arrow following ITR indicate the orientation. The vertical lines to the right of the P_{CMV} indicate restriction sites in the polylinker.

FIG. 4. Construction of a 'gutless' adenoviral vector. In the first step shuttle plasmid cleaved at the ITR is transfected into CRE8 cells with Ψ5 DNA.

The proteins from Ψ5 convert the shuttle plasmid DNA to a molecule which is replicated by adenovirus DNA polymerase. The gutless virus is then encapsidated into adenovirus capsids. Several rounds of growth are necessary to amplify the gutless virus. At each round, more Ψ5 virus is added to insure that all cells contain the helper virus.

FIG. 5. is a gel showing a restriction analysis of packaged gutless and Ψ5 DNA from infected CRE8 cells. The DNA was digested with Bgl II. There are no Bgl II sites in the loxAβ gutless virus. The lanes contain: M, 1 kb ladder + 16.5 and 33.5 kb fragments; Ψ5 DNA; lanes 1-6, isolates of loxAβ + Ψ5. The arrow marks the position of loxAβ DNA.

FIG. 6. is a gel showing a restriction analysis of loxAβ + Ψ5 DNA with ClaI. Ψ5 DNA contains one site at base 1473. The predicted sizes of the loxAβ fragments are 0.5, 1.2, 6, 7.5, and 11.4 kb. The lanes contain: M, 1 kb ladder; P, ploxAβ cut with ClaI; isolates 2, 5 and 6 from part a cut with ClaI. (Many of the bands from ploxAβ do not match up with their cognate bands in loxAβ as the ClaI sites in the plasmid are methylated.)

FIG. 7. Restriction analysis of Ψ5 mutants with BsaBI. The position of the left end fragment from Ψ5 is marked. The DNA was from 16 plaque isolates purified on 293 cells.

FIG. 8A-8C. Methods for selecting gutless viruses. These methods allow for enrichment of gutless virus starting with a mixture containing about 0.5 % gutless, and going to roughly 50 %. In each arrangement, the gutless virus will be enriched based on expression of a gene in the virus. This selectable marker is flanked with lox511 sites and will be deleted from the gutless virus by growth in CRE8 cells for the final step of enrichment. The final enrichment takes the gutless virus concentration to 95% or more. ITR is the inverted terminal repeat of an adenovirus, Ψ is the packaging site of an adenovirus. loxP and lox511 are loci of Cre recombinase directed recombination. loxP will recombine with loxP but not lox511 and visa versa.

FIG. 8A. Sorting for an expressed gene on the gutless virus. Here the selection is accomplished by mechanical means such as a cell sorter or by panning with an antibody. Ψ5 is used as a helper virus. Ψ5 DNA and gutless plasmid are

cotransfected into 293 cells. The viruses are then grown together in 293 cells until the final passage through CRE8 cells.

FIG. 8B. Enrichment by complementation. An adenovirus gene is inserted between the lox511 sites in the gutless virus, E4 for example. A helper virus which is an E4 deleted version of Ψ5 would then be cotransected into 293 cells. There should be no sequence in common flanking the E4 gene and the E4 deletion to minimize recombination between helper and gutless viruses. Only those cells containing both viruses will produce virus. The E4 deleted helper virus must be grown on a cell line complementing E1 and E4 genes.

FIG. 8C. A dominant selection in *trans*. A transcriptional regulator protein would be inserted between the lox511 sites in the gutless virus. The helper virus would be modified by placing a selectable marker in the E1 region controlled by the transcriptional regulator. The selection could be either positive or negative. For positive selection, a viral gene would be moved to the E1 region in the helper virus, fiber for example. Under this scenario, fiber gene transcription would require the regulatory protein from the gutless virus. For negative regulation, a poisonous gene such as herpes virus thymidine kinase (tk) would be inserted into the E1 region of the helper virus and gancyclovir added to the growth media. Here the regulatory protein would repress expression of the tk gene; otherwise the combination of tk and gancyclovir would poison viral DNA replication.

FIG. 9. Encapsidated loxβA 'gutless' and Ψ5 helper viruses on 293 and CRE8 cells. Both DNA's were digested with BglII. Two different amounts of each sample is shown.

FIG. 10A and 10B. Titration of a 1::1 mixture of loxβA and Ψ5 on CRE8 cells. Packaged DNA was prepared from 10^7 cells infected at moi's shown. The moi's are approximate and based on a single virus. **FIG. 10A:** The * on the gel marks the position of a 2.2 kb fragment unique to the helper virus. The major band migrating at 2.8 kb is from a pair of loxβA fragments. **FIG. 10B:** the graph shows the result of band intensities of the 2.2 and 2.8 kb bands, corrected for size and molarity.

FIG. 11A and 11B. Effect of DNA phasing on Cre/lox recombination. The Ψ9 and Ψ9+17 viruses were mixed with Ad β-gal virus and used to infect either 293 or CRE8 cells. Packaged DNA was prepared, digested with BsaBI, separated in an agarose gel and the intensities of the labeled bands were determined. **FIG. 11A** is a photograph of a gel showing BsaBI-digested DNAs. **FIG. 11B** is a diagram showing the phasing of the lox sites in the two different viruses, Ψ9 and Ψ9+17.

FIG. 12A and 12B. Relative packaging efficiency of Ψ11 virus. A mixture of 20 parts Ψ11 to one part dl309 was used to infect 10^7 either 293 or CRE8 cells at an moi of 10. Encapsidated DNA was prepared and 1/25 of the DNA was subjected to 10 cycles of PCR with primers which recognized both viral DNA's. There was no product when the viral DNA's were omitted. **FIG. 12A** is a gel showing the results. **FIG. 12B** is a diagram showing the structures of Ad5 and ψ11.

FIG. 13A and 13B. Relative packaging efficiency of Ψ7 virus. Equal numbers of particles of Ψ7 and Ad tet β-gal were mixed and used to infect 10^7 293 or CRE8 cells at an moi of 10 for each virus. **FIG. 13A:** Packaged DNA was digested with BsaBI, separated and the intensities of the left end fragments were determined. **FIG. 13B:** In a measurement of the relative encapsidation efficiency, Ψ7 was encapsidated at 30 and 5.9% of a similar virus with a normal Ad5 packaging site when grown in 293 and CRE8 cells respectively.

FIG. 14. Construction of an E1-substituted adenovirus by using Ψ5 and a replicating vector. Negative pressure on Ψ5 is achieved by intramolecular recombination removing the packaging site in the first step. An intermolecular recombination between the replicating vector and Ψ5 then creates a new virus which has an intact packaging site and carries a recombinant gene, marked Exp. Cassette. The packaging site is labeled Ψ.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention provides an integrated system for the *in vivo* production of substantially pure preparations of packaged adenovirus-based vectors useful for gene therapy and gene transfer and expression in mammalian cells. The

system has several components including a therapeutic recombinant adenovirus vector, a helper recombinant adenovirus vector and a host cell line which expresses Cre recombinase. The present invention provides each of these components individually as well as a method for production of *in vivo* packaged therapeutic rAd vectors using the system.

In brief, the method of the present invention works as follows. A eukaryotic host cell which is susceptible to adenovirus infection is transfected with a therapeutic rAd vector and a helper rAd vector. The therapeutic rAd vector is replicated and packaged in the transfected host by the replication and packaging system that is supplied by the helper virus. The therapeutic rAd vector contains Ad DNA sequences only from the ITRs and the packaging site, the remainder of the DNA in the therapeutic rAd vector is from non-adenovirus sources. The helper rAd vector is constructed so that the packaging site is flanked by *lox* sites which recombine with great efficiency in the presence of Cre recombinase. The recombination of the *lox* sites results in the excision of the packaging site. The efficiency of the excision of the packaging site in the helper can be improved by using more than one set of *lox* sites flanking the packaging site. The system contains the further safeguard that any homologous recombination between the helper rAd vector and the therapeutic rAd vector (whether Cre-mediated or not) can only result in a helper rAd vector in which the packaging site is still flanked by *lox* sites and thus still vulnerable to excision. The transfection produces a mixture of packaged particles, both therapeutic rAd vector particles and helper rAd vector particles. The packaged viral particles from the transfection are isolated by standard methods and used to infect additional host cells. The infection steps may be repeated a number of times until the titer of the viral particles is produced in sufficient quantities. In the final infection step, host cells capable of expressing the Cre recombinase are used. Cre-expressing host cells may be used in all of the transfection and infection steps as well, however, for infection and transfection steps other than the final one, non-Cre expressing cells are preferred because of the possibility that prolonged selective pressure will result in unwanted deletions in the helper rAd vector.

For ease of description of the rAd vectors of the present invention, reference will be made to the genetic map of adenovirus in Figure 1. The terms "left" and "right" are defined with reference to the structure of adenovirus as it appears in Figure 1. The terms "3′" and "5′" are defined with reference to the upper DNA strand as shown in Figure 1. The rAd vectors of the present invention, including the therapeutic rAd vector and the helper rAd vector, while different in some respects from the wild type adenovirus genetic map in Figure 1, still retain certain adenovirus structural landmarks (e.g. ITRs, packaging site) so that the left/right designation retains the same meaning for the rAd vectors as for wild type Ad. The complete sequence of the wild type Ad5 virus is known and can be found in Chroboczek *et al.* Virology 186:280-285 (1992), GENBANK Accession No. M73260.

The therapeutic rAd vectors of the present invention comprise DNA molecules containing adenovirus inverted terminal repeats (ITRs), an adenovirus packaging site and one or more *lox* sites. The therapeutic rAd vectors additionally comprise a foreign DNA sequence of interest. The ITRs and the packaging site are required *in cis* for replication and packaging of the therapeutic vectors *in vivo*. Adenovirus ITRs useful for the therapeutic rAd vectors of the present invention can be the ITRs from any adenovirus as long as they are recognized by the replication and packaging proteins supplied *in trans* by the helper rAd vector. All naturally occurring adenoviruses have inverted terminal repeats although the length and sequence of the ITRs vary among different adenovirus serotypes. The sequences of many of the ITRs are known (see for example, Sussenbach, J. in "The Adenoviruses" pages 35-113 Ginsberg, H. ed. Plenum Press 1984). Preferably, the ITRs in the therapeutic rAd vectors are the ITRs from Ad2, Ad3, Ad4, Ad5, Ad7, Ad9, Ad10, Ad12, Ad18 or Ad31. More preferably the ITRs are from Ad5. The ITRs are oriented in the therapeutic rAd vectors in the same manner in which they are oriented in the naturally occurring adenovirus, that is, the sequences are inverted with respect to one another and occur at the terminals of the therapeutic rAd vectors. In the therapeutic rAd vectors, the ITRs are separated by the *lox* site or sites, the packaging site, and the foreign DNA sequence of interest.

The adenovirus packaging site is required *in cis* for packaging of the DNA into the adenovirus virions. All adenovirus strains analyzed to date contain a packaging site, typically located at the left end of the viral genome, adjacent to and to the right of the left ITR. In addition to the naturally-occurring Ad packaging sites, certain other DNA sequences have been shown empirically to function as packaging sites. Such sequences are referred to as synthetic packaging sites. For the therapeutic rAd vectors of the present invention, any naturally-occurring or synthetic adenovirus packaging site is suitable as long as the site is recognized by the packaging system of the helper rAd virus used. Preferably, the packaging site is that from Ad5. In particular, the Ad5 packaging site is the DNA sequence from base pair 194 to base pair 452 of the Ad5 genome as measured from the left end. More preferably, the packaging site is the DNA sequence from base pair 194 to base pair 375 of the Ad5 genome as measured from the left end. Alternatively, the packaging site useful for the therapeutic rAd vectors of the present invention may be a synthetic packaging site. For example, one synthetic packaging site composed of six tandemly repeated copies of the "A" repeat has been shown the function as a packaging site *in vivo* (Grable and Hearing J. Virol. 64:2047-2056). Preferably, for the therapeutic rAd vectors of the present invention, the packaging site is a naturally-occurring adenovirus packaging site.

The therapeutic rAd vectors of the present invention contain at least one *lox* site for recombination. *Lox* sites are the sites at which Cre-mediated recombination occurs. The presence of at least one *lox* site in the therapeutic rAd vector of the same type as at least one of the *lox* sites in the helper rAd vector serves to insure that any homologous recombination between the therapeutic rAd vector and the helper rAd vector (through the ITRs or packaging sites, for example) can only result in helper rAd vectors which still retain *lox* sites flanking the packaging site. Several types of *lox* sites have been identified and their sequences are well known. Any *lox* site that is capable of undergoing Cre-mediated recombination is suitable for use in the therapeutic rAd vector. Preferably for the therapeutic rAd vectors of the present invention, one or more of the following *lox* sites are useful: *lox* P, *lox* 511, *lox* 514 and *lox* Psym (Hoess *et al*. Nucl. Acids Res. 14:2287-2301 1986). The sequence of several *lox* sites are

shown in Figure 2. The therapeutic rAd vectors of the present invention may contain one or more types of *lox* sites but will contain no more than one *lox* site of each type. At least one of the *lox* site(s) used in the therapeutic rAD vector will be of the same type as at least one of the *lox* sites used in the helper rAd vector as described below.

The therapeutic rAd vectors of the present invention contain a foreign DNA sequence of interest. The foreign DNA sequence of interest typically comprises genes or other DNA sequences that are of interest to transfer into mammalian cells. Foreign DNA sequence of interest may include any naturally occurring or synthetic DNA sequence. The foreign DNA may encode protein, or contain regulatory sites, including but not limited to, transcription factor binding sites, promoters, enhancers, silencers, ribosome binding sequences, recombination sites, origins of replication, sequences which regulate RNA stability and polyadenylation signals. The foreign DNA may be identical in sequence to naturally-occurring DNA or may be mutated relative to the naturally occurring sequence. The foreign DNA need not be characterized as to sequence or function.

The size of foreign DNA that may be included in the therapeutic rAd vector will depend upon the size of the rest of the vector. Preferably, the total size of foreign DNA is from 36 kb to 37.4 kb. The total size of the therapeutic rAd vector will be not larger than about 38 kb. Preferably, the total size of the therapeutic rAd vector is from 32 to 38 kb; more preferably, from 34 kb to 37 kb; and most preferably from 35 kb to 36 kb. The lower size limit for packaging efficiency (i.e. at least 80% of wild type packaging efficiency) is around 32 kb total rAd vector. Recombinant Ad viruses that are smaller than 32 kb are not as stable, which can create significant problems for reliable delivery of the same recombinant vector for gene therapy. This discovery of the optimum packaging size is also important for manufacturing large quantities of the viral particles for commercial use, in which case packaging efficiencies on the order of wild type efficiencies are desired.

The construction of the therapeutic rAd vector of the present invention is accomplished by operationally joining the various required DNA sequences that comprise the therapeutic rAd vector, that is, the left and right ITR sequences, the

adenovirus packaging site sequence, the *lox* site sequence(s) and the foreign DNA sequence of interest. The various required DNA sequences are joined together in a particular order and in a specific orientation with regard to one another. The order and orientation of the sequences is illustrated from the left to right direction as follows: a left inverted terminal repeat, one or more *lox* recombination sites, an adenovirus packaging site, a foreign DNA sequence of interest, a right inverted terminal repeat. The required sequences may be joined directly to one another or additional DNA sequences may intervene between the required sequences. The additional intervening DNA sequences may be residual from the cloning process used to join the required sequences or may be particular sequences positioned between or within the required DNA sequences in order to aid in manipulation or efficiency of the vectors, for example, restriction sites, PCR priming sites, promoters, selectable marker genes and the like. The left and right inverted terminal repeats are oriented toward one another in the therapeutic rAd vector in the same manner as that in which they are found naturally-occurring in the adenovirus, that is they are inversely oriented with respect to one another. By inversely oriented is meant that when read on the same strand in the 5′ to 3′ direction, the ITR sequences are the reverse complements of one another. The orientation of the packaging site is not critical for the vectors of the present invention so that the packaging site may be oriented in either direction with respect to the left ITR. The packaging site will be positioned no more than 400 bp from the left end of the therapeutic rAd vector, preferably no more than 300 bp from the left end of the vector. The *lox* site is oriented with respect to the packaging site in the same manner as are the identical *lox* sites in the helper rAd with respect to the packaging site, as described below. If more than one type of *lox* site is present, the *lox* sites may preferably be ordered in the same manner as they occur on the left side of the packaging site in the particular helper rAd vector to be used with the therapeutic rAd vector. The foreign DNA of interest may be oriented in any appropriate orientation with respect to the rest of the vector. The sequences are joined together by any of a number of techniques that are well known in the DNA cloning art. The joining is most conveniently accomplished by ligation of DNA fragments, for instance restriction fragments or chemically or

enzymatically synthesized DNA fragments, containing the various required DNA sequences. Alternatively, the various required DNA sequences may be chemically or enzymatically synthesized as a single DNA fragment or two or more of the various required sequences may be synthesized as a single fragment and joined to the remaining sequences.

As an alternative to *in vitro* vector construction methods described above, the therapeutic rAd may be constructed *in vivo* by homologous recombination techniques that are well known in the art (see for example, Chinniduri *et al.* J. Virol. 32:623-628 (1979); Bett *et al.* (1994)). *In vivo* construction using Cre-mediated recombination of *lox* sites may also be used to create the therapeutic rAd vector. For this purpose, a precursor therapeutic rAd vector may be constructed without ITR sequences and containing an additional *lox* site, located at the right-most end of the foreign DNA. The precursor therapeutic rAd vector thus has the following sequences in order: a first *lox* site, an Ad packaging site, the foreign DNA, and a second *lox* site not identical to the first site. A modified helper rAd vector is constructed as described below except that an additional *lox* site, identical to the second *lox* site in the precursor therapeutic rAd vector, is positioned adjacent to and to the left of the right ITR. The additional *lox* site is not identical to any of the other *lox* sites in the modified helper. The precursor therapeutic rAd vector and the modified helper rAd are transfected into a Cre-expressing host cell and Cre-mediated recombination results in a transfer of the ITRs from the modified helper to the precursor therapeutic rAd to form a therapeutic rAd vector.

The therapeutic rAd vector may be conveniently cloned into a prokaryotic cloning vehicle (for example, plasmids, bacteriophages, phagemids, etc.) for easy propagation in a bacterial host. Alternatively, a eukaryotic cloning vehicle may be used. For this purpose, restriction sites may be added to the outer most ends of the ITRs. Prior to use for transfection, the therapeutic rAd vector is removed from the cloning vehicle by appropriate restriction digestion. It will be apparent that the therapeutic rAd vector may be constructed first and then cloned into a cloning vehicle or each sequence required in the therapeutic rAd vector may be added separately to the cloning vehicle. Methods of vector construction are well known in the art and one of ordinary skill would readily be able to determine

an appropriate cloning strategy for construction of the therapeutic rAd of the present invention either with or without a cloning vehicle.

The helper rAd vector of the present invention comprises a DNA molecule containing adenovirus genes which encode proteins necessary for the replication and packaging of the therapeutic rAd vectors into therapeutic rAd virus particles. The helper rAd vector of the present invention additionally comprises an adenovirus packaging site, flanked by at least one set of two identical *lox* sites in direct orientation with respect to one another, and a left and a right ITR. The helper rAd vector may contain an entire adenovirus genome provided that any packaging site is flanked by at least one set of two directly-oriented, identical *lox* sites. Particular adenovirus genes may be omitted from the helper rAd virus if the products of the omitted genes are not essential for the replication and packaging of the therapeutic rAd (for example, the E3 proteins, E4 or 1-4 or VAII) or can be supplied otherwise than from the helper rAd vector, for example, from the host cell. The determination of which Ad genes may be omitted from the helper rAd is well within the ability of one of ordinary skill in the art. Preferably, the helper rAd vector will contain the entire adenovirus genome except for the E1a and E1b regions. When the helper rAd vector does not contain the E1a and E1b regions, it is preferably used in combination with a host cell in which the E1a and E1b gene products are supplied from the host. The E1a and E1b gene products are preferably transcribed from heterologous promoters in the host cell.

The adenovirus packaging site suitable for the helper rAd vector of the present invention includes any of the packaging sites that are useful for the therapeutic rAd vector. Preferably, a synthetic packaging site is useful for the helper rAd vector. In general, Ad vectors containing the synthetic packaging sites are less efficiently packaged. Use of such a site therefore contributes to the selection against the helper rAd. Most preferably, the packaging site comprises a DNA sequence of six tandemly repeated "A" repeats (Grable and Hearing, J. Virol. 64:2047-2056).

The packaging site of the helper rAd vector is flanked by at least one set of two identical *lox* sites in direct orientation with respect to one another, one of the set being on the left (5'-most) side of the packaging site and one of the set being

on the right (3'-most) side of the packaging site. By direct orientation is meant that the sequences of the *lox* sites when read in the 5' to 3' direction on the same DNA strand are identical. When more than one set of two identical *lox* sites is present in the helper rAd vector they will be arranged so as to form a nested set; that is, one member of each set will be positioned on the left side of the packaging site and one member will be positioned on the right side of the packaging site and the order of the sites as they occur on the right side of the packaging site will be opposite to that in which they occur on the left side but the two identical members of each set will be in the direct orientation with respect to one another.

One such nested set may be illustrated by a simple example. A helper rAd having three sets of two identical *lox* sites, for instance one set each of *lox* P, *lox* 511 and *lox* 514, may be arranged in order ....*lox* P(→)-*lox* 511(→)-*lox* 514(→)-packaging site-*lox* 514(→)-*lox* 511(→)-*lox* P(→).... where the arrows indicate the directionality of the *lox* sites.

Where more than one set of two identical *lox* sites are used, the identical innermost lox sites will preferably be separated from one another by at least 60 base pairs of spacer DNA, wherein the identical lox sites are in phase with each other (i.e. the distance between the identical lox sites before the recombinase/excision step is a multiple of 10.5 base pairs). The lox sites should also be preferably at least 14 bases apart from the adjacent, non-identical lox sites. The distances between lox sites can be created by addition of spacer DNA. The addition of spacer DNA between the *lox* sites insures that there will be a sufficient amount of intervening DNA between the remaining identical *lox* sites for recombination to occur after recombination has occurred between the innermost *lox* sites in the nested set. The center-to-center distance between the innermost nested lox sites will contain the packaging site, which is greater than the minimal size between lox sites that is required for excision by recombinase--roughly 60 base pairs. The center-to-center distance between the innermost lox sites preferably will be the sum of the size of the packaging site plus additional spacer DNA such that the sum is a multiple of 10.5. The center-to-center distance being a multiple of 10.5 base pairs in length places the lox sites in phase with each other, since there are roughly 10.5 bases per helical turn. The second set of

nested lox sites, flanking the innermost set, should comprise two different lox sites, each preferably at least 14 bases distant from the center of the adjacent, non-identical innermost lox site. The distance between the second set of nested lox sites and the adjacent, non-identical innermost lox sites can be optimized by calculating a distance between the second set of nested lox sites that is a multiple of 10.5 bases pairs after excision of the innermost lox sites, wherein the excision leaves a single innermost lox site and spacer DNA. The phasing of lox sites can also be calculated in designing the third set of flanking lox sites in a nested set lox site configuration.

The spacer DNA inserted between the lox sites may also enhance the Cre mediated recombination. In general, it appears that the spacer sequence may be nonspecific and only its length of importance. For instance, interaction between proteins at two different sites on DNA is maximal when the proteins are in phase, that is on the same face of the DNA helix. There are about 10 base pairs per turn of the DNA helix. Additionally, since the helper virus DNA may be in nucleosomes the phasing of the sites for maximal recombination may reflect nucleosomal phasing of 160 to 200 base pairs. However, some aspects of the sequence in the spacer may also be important. As is well known in the art, certain sequences bend DNA or alter its stiffness. In addition, DNA binding proteins can bend DNA and some DNA binding proteins can displace or phase nucleosomes. The spacer DNA may separate the *lox* sites on the right side of the packaging site or those on the left side of the packaging site or both. Preferably, the spacer DNA will separate the *lox* sites on the right side of the packaging site. As in the therapeutic rAd vector, the packaging site in the helper rAd vector will be no more than 400 base pairs, preferably no more than 300 base pairs, from the left ITR.

The left and right ITRs useful for the helper rAd vector can be the ITRs from any adenovirus as long as they are recognized by replication and packaging proteins expressed by the helper rAd vector. Preferably, the ITRs in the helper rAd vectors are the ITRs from Ad2, Ad3, Ad4, Ad5, Ad7, Ad9, Ad10, Ad12, Ad18 or Ad31. More preferably the ITRs are from Ad5. The ITRs are oriented in the helper rAd vectors in the same manner in which they are oriented in the

naturally occurring adenovirus, that is, the sequences are inverted with respect to one another and occur at the terminals of the helper rAd vectors. In the helper rAd vectors, the ITRs are separated by the packaging site, which itself is flanked by at least one identical set of *lox* sites, and adenoviral DNA which encodes the proteins necessary for replication and packaging.

The helper rAd vector may be constructed using any of a number of cloning techniques well known in the art. Such techniques have been described for the construction of the therapeutic rAd vector and include *in vitro* cloning, *in vivo* recombination, chemical or enzymatic synthesis, and any other appropriate methods.

A Cre-expressing host cell culture for use in the present invention can be made in any of a number of ways that are well known in the art. The Cre expressing host cell may be made by transfection of a mammalian cell culture that is susceptible to infection by adenovirus with a DNA vector containing a functional Cre gene or coding sequence. By functional is meant that the gene or coding sequence also contains those regulatory sequences necessary for transcription, translation and localization in the cell into which it is delivered such that Cre is expressed. For example, Adenovirus susceptible cells may be transfected with a vector carrying the Cre gene from bacteriophage P1, under control of a eukaryotic promoter, for example the immediate-early promoter of CMV. The bacteriophage Cre gene may be modified, using techniques that are well known in the art, to include a Kozak sequence and a nuclear localization signal for maximum translational efficiency and transport into the nucleus. Transfectants may be assayed for Cre activity by any conventional method including Western blots with Cre-specific antibody or functional assays for protein activity (see, for example, Adams *et al.* J. Mol. Biol. 226:661-673). Typically, for use in the present invention, Cre-expressing host cell culture will produce an intracellular Cre concentration of between 0.1μM and 50 μM, preferably between 1μM and 20μM. Preferably, the Cre-expressing host cell culture will also express Ela and E1b and the cell will not contain the adenovirus packaging site.

In addition, the chromosomal or episomal DNA of the Cre-expressing host cell

preferably does not contain any sequences capable of recombination with the helper rAd vector.

The method of the present invention provides for the production of a substantially pure preparation of packaged therapeutic rAd vector particles. By a packaged therapeutic rAd vector particle is meant a therapeutic rAd vector DNA packaged into an adenovirus virion to form an infectious particle. The packaged therapeutic rAd vector particles may also be referred to as therapeutic rAd virus. By infectious is meant that the packaged particle is at least capable of binding to the high affinity Ad receptor on the host cell, followed by internalization and transport of the DNA to the nucleus. By substantially pure is meant a preparation in which at least 95% of the vector particles present are therapeutic rAd vector particles; preferably at least 99% of the vector particles present are therapeutic rAd vector particles, more preferably at least 99.5% of the vector particles present are therapeutic rAd vector particles.

The method of the present invention is initiated by transfecting a host cell susceptible to adenovirus infection with a therapeutic rAd vector and a helper rAd vector. The host cell preferably does not contain any chromosomal or episomal DNA which is capable of recombination with the helper rAd vector. The host cell used for transfection may be any cell culture susceptible to Adenovirus infection. The host may be one which is capable of expressing Cre recombinase, but non-Cre-expressing host cells are preferred for the transfection step. The choice of a particular therapeutic rAd vector and a particular helper rAd vector is limited only by two requirements: (1) that the replication and packaging proteins encoded by the helper rAd vector must be able to recognize the ITRs of the helper rAd vector and the ITRs and packaging site of the therapeutic rAd vector and (2) that the therapeutic rAd vector must contain at least one *lox* site to the left of the packaging site that is identical to one of the *lox* sites in the equivalent position in the helper rAd vector. If the rAd vectors have been cloned into and propagated in cloning vehicles, the cloning vehicle DNA may be removed prior to transfection such that only the rAd vector is used. The cloning vehicle DNA may be removed in any of a number of ways that are well known in the art, for example by

restriction digestion or by synthesis of PCR fragments containing only the rAd vector sequences.

Transfection may be performed by the DEAE-dextran method (McCutchen and Pagano, 1968, J. Natl. Cancer Inst. 41:351-357), the calcium phosphate procedure (Graham *et al.*, J. Virol. 33:739-748 (1973); Graham and van der Eb, Virology 52:456-467 (1973)) or by any other method known in the art, including but not limited to microinjection, lipofection, and electroporation. In most cases, the cells will be transfected with the helper rAd and the therapeutic rAd simultaneously, although there may be instances in which it is more appropriate to transfect with each vector separately. Typically, an equimolar amount of the helper rAd vector and the therapeutic rAd vector will be used but this may be varied for optimal yields depending on the vectors used. Determination of the appropriate ratio for transfection is well within the skill of one of ordinary skill in the art.

The transfected cells are cultured in a suitable medium, for example DMEM, for a time sufficient to provide for maximum replication and packaging of the therapeutic rAd vectors into viral particles. The point of maximum replication and packaging of the therapeutic rAd can be estimated by determining the viral yield at various times after transfection. The viral yield can be determined by conventional methods, including infection of Ad susceptible host with lysates and counting the number of infected cells expressing a marker gene contained in the foreign DNA, quantitative PCR for sequences unique to the therapeutic rAd, measurement of absorbance of the viral preparation at 280 nm after correcting for any helper virus that might be present (the titer of any contaminating helper rAd virus is easily determined by plaque assays), or by measurement of absorbance of the viral DNA at 260 nm (any contamination with helper rAd DNA can be determined by appropriate restriction digestion). In most cases, 72 hours is a sufficient time period for culturing the transfected cells. Following the transfection and during the culturing period, the helper rAd is transcribed and translated to produce the adenovirus gene products necessary for the replication and packaging of the therapeutic rAd.

The packaged vector particles are isolated from the transfected cells by conventional means. Conventional means includes producing a lysate of the whole plate or overlaying the monolayer of cells with agar and then removing only the infected cells in the plaques. Plaques are made of dead infected cells and are visible to the eye. The plaques may also be stained for expression of a marker gene in the rAd virus. Plaque isolation is typically done at about a week after transfection. The vector particles isolated from the transfected cells are a mixture of packaged therapeutic rAd vector particles and packaged helper rAd vector particles. The mixture of vector particles isolated from the transfected cells is used to infect a fresh host cell culture by methods that are well known in the art. Packaged viral particles are isolated from the infected cells and the infection process is repeated with fresh host cells in order to amplify the viral titer with each passage. Since the yield of rAd virus from the transfection is quite small, it is important to infect a small number of cells (10^2). At each passage the cell number is increased 10^2 to 10^4 to 10^6 to 10^8. Because infection with the packaged vector particles is much more efficient than transfection with the naked vector DNA, more of the cells will receive and replicate the vectors in the infection steps.

As in the transfection, the co-infection yields a mixture of packaged rAd particles. The packaged rAd vector particles are isolated from the infected cells in the same manner as from the transfected cells and may be used to repeat the infection steps as many times as necessary to produce a sufficient titer of substantially pure packaged therapeutic rAd vector particles. In general the titer will be between 10^7 and 10^{10} per ml, preferably between 10^9 and 10^{10} per ml.

In the final step of the method of the present invention, a Cre-expressing cell line is used as the host cell for infection. In the Cre-expressing cells, the helper rAd undergoes replication but the packaging of the helper rAd will be limited because Cre-mediated recombination between the *lox* sites will result in the excision of the packaging site from a large percentage of the helper rAd molecules. Helper rAd without the packaging site is not packaged into virions. When one set of *lox* sites is present in the helper rAd, packaging site will be excised in about 95% of the molecules; when two sets of *lox* sites are used, the

molecules with an excised packaging site increases to about 99%; when three sets are used the percentage increases to about 99.99%. In addition, any host-mediated recombination that may occur between the therapeutic rAd vector and the helper rAd vector (for example, due to the possible homology of the ITRs or the packaging sites) during the replication and packaging process will not result in regeneration of a wild type adenovirus. Because the therapeutic rAd vector contains a *lox* site between the left ITR and the packaging site, any recombination with the helper rAd will result only in a helper vector in which the packaging site is still flanked by at least one set of identical *lox* sites as long as the *lox* site in the therapeutic virus is identical to at least one of the *lox* sites in the helper rAd vector.

Typically the method of the present invention is carried out as follows. The therapeutic rAd vector and the helper rAd vector are transfected into non-Cre-expressing cells (that is, non-selective cells). The monolayer of transfected cells is overlain with agar and plaques are removed after one week. The virus mixture from the plaques is passed successively by infection onto increasing numbers of non-Cre-expressing (non-selective) cells; 10^7 non-Cre-expressing cells are infected and the resulting viral DNA is screened for the presence of the rAd virus. The helper virus is reduced in the final passage by infecting Cre recombinase expressing cells. By limiting passage of the helper rAd through Cre-expressing host cells to the final infection step, helper virus is not under continuous selection and the possibility of mutations rendering the helper resistant to Cre selection is lessened.

In another embodiment, the present invention comprises a method for rapidly and efficiently generating new recombinant adenovirus vectors with substitutions in the E1 region or any other adenoviral region. This method uses a helper rAd of the present invention, for example Ψ5, and Cre-expressing host cells to generate new adenovirus recombinants *in vivo*. The helper vector comprises a packaging site flanked by recombination sites and an ITR, wherein the packaging site can be located at either end of the helper vector. The helper vector can also contain adenoviral or foreign DNA sequences between the recombination sites, wherein the adenoviral or foreign DNA sequences can be excised or deleted via

recombinase-mediated recombination. The adenoviral DNA to be deleted can be from any region of the adenoviral genome expressing genes that can be complemented. One advantage of deleting adenoviral or foreign DNA sequences is to provide room for substitute DNA. The helper rAd vector is transfected or infected into Cre-expressing host cells along with a replicating vector containing the Ad ITRs separated by an Ad packaging site, the substitute DNA to be substituted into the E1 region (or any region in which a substitution is preferred) and a *lox* site identical to at least one of the *lox* sites in the helper rAd vector. During the growth of the virus in the transfected cells, the packaging site in the helper rAd is excised by Cre-mediated recombination to yield a deleted helper rAd (Δ-helper) containing no packaging site and only a single *lox* site. Recombination of the Δ-helper with the replicating vector at the *lox* site yields packageable virus containing the essential viral genes from the Δ-helper and the substitute DNA from the replicating vector. If the substitute DNA includes adenoviral genes that are necessary for replication, then the resulting substituted rAd vector will be a replicating virus. On the other hand, if the substitute DNA does not introduce adenoviral genes that are necessary for replication and are not otherwise available, then the resulting substituted rAd vector will be a nonreplicating virus.

One of ordinary skill in the art will appreciate that this method can be used to generate new rAd vectors with substitutions in any Ad region, including but not limited to the E1 region.

By replicating vector is meant any vector that can be replicated by the adenovirus replication system. The replicating vector must contain Ad inverted terminal repeats. The replicating vector will also contain an Ad packaging site and a *lox* site. Preferably the replicating vector will additionally contain substitute DNA inserted between the packaging site and the *lox* site. The replicating vector is preferably pAd*lox* or pAd*lox* derivatives having the substitute DNA inserted in the polylinker region. By substitute DNA is meant any DNA to be substituted into the E1 or other Ad region.

By using selection against Ψ5, a recombinant adenovirus carrying substitute DNA in place of the E1 genes was generated by cotransfecting a replicating vector with a *loxP* site (pAdlox) and Ψ5 DNA into a CRE8 cells (Fig. 14). In the first

step of the reaction, Cre recombinase catalyzes recombination between the two *loxP* sites in Ψ5, removing the packaging site from the virus. In the second step, Cre recombinase catalyzes a recombination between Ψ5 and pAdlox, transferring the substitute DNA into Ψ5. The resulting recombinant virus will now have a single *loxP* site and therefore will have a considerable growth advantage over Ψ5 in CRE8 cells. This growth advantage should generate virus stocks comprised predominantly of the recombinant adenovirus having a substitution.

Specific examples of the steps described above are set forth in the following examples. However, it will be apparent to one of ordinary skill in the art that many modifications are possible and that the examples are provided for purposes of illustration only and are not limiting of the invention unless so specified.

EXAMPLES

EXAMPLE 1. CONSTRUCTION OF THE Ψ5 HELPER RAD VECTOR

The starting material for construction of the helper rAd vector Ψ5 was a plasmid containing the following sequences in order: an SfiI restriction site, nucleotides 2 to 553 from Ad5 (nucleotide 1 is the last C of the SfiI site), an XhoI restriction site, the human CMV immediate-early promoter from -600 to +1 relative to the start of transcription, the polylinker sequence from pSP73 from the HindIII site to the EcoRI site, a polyadenylation signal from SV40 (nucleotides 2752 to 2534 of SV40), pSP73 sequence from nucleotide 2 through 2382 (containing ClaI, EcoRV, and BglII sites), an ApaI restriction site, the right ITR from Ad5. The ApaI-SfiI fragment containing the right ITR was made by polymerase chain reaction. For ease of manipulation the SfiI fragment was cloned into the PvuII site of pBluescript$^+$ to give pCMV-Ad. Cleavage of pCMV-Ad with SfiI releases the original SfiI fragment.

Next, a single *lox* P site (chemically synthesized) was inserted into pCMV-Ad between the ClaI and BglII sites to give plasmid pAd*lox*. A second *lox* P site was inserted into pAd*lox* between nucleotides 193 and 194 of the Ad5 left end fragment to give pf*lox*Pac. The orientation of the two *lox* P sites in pf*lox*Pac is

the same. Finally, nucleotides 3328 through 8914 of Ad5 were inserted into the BglII site of pf*lox*Pac to create pf*lox*PacB.

Ψ5 was made by overlap recombination between SfiI cleaved pf*lox*PacB and ClaI cleaved Adβgal in 293 cells using $CaPO_4$ transfection (Chinniduri *et al.* J. Virol. 32:623-628 (1979)). Adβgal contains wild type adenovirus sequence from nucleotide 3328 through the right end except for a deletion between nucleotides 28,592 and 30,470 in the E3 region. The resulting recombinant virus was isolated by plaque purification using standard techniques. The structure of Ψ5 is shown in Figure 3.

EXAMPLE 2. CONSTRUCTION OF A THERAPEUTIC RAD VECTOR MINUS *LOX* SITE.

A minimal rAd vector was constructed containing the ITRs, Ad packaging site and about 27 kb of foreign DNA. This prototype therapeutic rAd vector did not contain a *lox* site.

The starting material was pCMV Ad described in Example 1. The 22kb BglII A fragment from λ phage DNA was inserted between the BamHI and the BglII sites of pCMV Ad. A 5 kb BamHI DNA fragment containing a CMV immediate early promoter, the β-galactosidase gene and SV40 polyadenylation signal was also inserted into the BglII site. The resulting plasmid, pAβ, contains a 28kb DNA fragment, including an Ad packaging site, bounded by two ITRs. The ITR bounded fragment can be excised by SfiI digestion.

To determine whether this vector would be replicated and packaged *in vivo* with a helper virus, pAβ treated in various ways was co-transfected with Adenovirus DNA into 293 cells. The treatments were as follows: 1) pAβ transfected as circular plasmid, 2) pAβ was digested with PstI before transfection (PstI digestion excises the β-gal expression cassette from the ITRs and the packaging site), 3) pAβ was digested with SfiI before transfection (SfiI digestion excises the entire ITR bounded fragment). Three μg of treated plasmid and 3 μg of adenovirus DNA were transfected into 293 cells by $CaPO_4$ precipitation. As a control, 3μg of a β-galactosidase expressing plasmid was co-transfected with 3 μg Adenovirus DNA. Three days after transfection, virus was harvested by three

cycles of freeze-thawing and 0.2 ml of each lysate was used to infect 293 cells. The infected cells were scored for β-galactosidase activity. The results were as follows: 1) uncut pAβ, 100/ml; 2) PstI cut pAβ; 200/ml; 3) SfiI cut pAβ, 2000/ml; 4) control β-galactosidase expression plasmid, 100/ml.

EXAMPLE 3. ISOLATION OF CRE8 CRE-EXPRESSING HOST CELL LINE.

To make a stable cell line expressing Cre recombinase, 293 cells were transfected with plasmid pML78 using the $CaPO_4$ precipitation method (Graham *et al.* Virology 52:456-467 (1973)). pML78 contains the Cre recombinase gene, modified to include a Kozak sequence at the start of translation and a nuclear localization signal at the N-terminal, under the control of a human β-actin promoter and followed by the poly A signal from β-actin. pML78 also contains a selectable neomycin resistance gene.

At 48 hr. after transfection, the cells were transferred into culture medium containing G418. After selection on G418 for 17 days, 12 resistant colonies were recovered and expanded.

As a first step in characterization of the transfectants, Western blot analysis was performed on cell extracts. A 10 cm dish of cells (about 1.6 X 10^7 cells) was washed once with phosphate buffered saline (PBS) and the cells were suspended in 5 ml PBS and centrifuged for 1 min. The cell pellet was resuspended in 400 μl extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.25% Triton X-100, 0.5 mM PMSF) and the cells were lysed by passing through a pipet tip 20 times. The lysate was gently mixed at 4° C for 30 min. and cleared by centrifugation for 3 min. in a microfuge at 4° C. The protein concentration of each extract was measured and an aliquot containing 200 μg protein was electrophoretically separated on a 10 % polyacrylamide gel containing SDS. The proteins were transferred to a nitrocellulose membrane and the Cre recombinase was identified using a monoclonal antibody against Cre (Sauer *et al.* Mol. Cell Biol. 7:2087-2096 (1987)). Ten of the cell lines tested had an immunologically reactive signal with an apparent migration corresponding to 38 kd. Control extract from 293 cells produced no signal at 38 kd. The concentration of Cre varied from 0.03 to 4 μM in the extracts. Assuming that one half of the volume of the cell is

taken up by the nucleus, this corresponds to a nuclear Cre concentration of 30 μM in the cell lines with the highest concentration.

To determine if the Cre protein detected in the Western blot was functional, a recombination assay was carried out as follows. Each cell line was transfected with a plasmid carrying the β-galactosidase gene flanked by non-equivalent *lox* sites (pAd*lox*2β-gal). Approximately 24 hrs later the transfected cell cultures were infected with an Adenovirus (Ad*lox*2) carrying the same two non-equivalent *lox* sites as pAd*lox*2β-gal. If recombination between the plasmid and the virus occurs at both *lox* sites, a new adenoviral vector carrying the β-galactosidase marker will be produced. Two days after infection the virus produced from each cell line was harvested and used to infect cells for detection of β-galactosidase activity by X-gal staining. The amount of recombinant virus produced in each cell line correlated well with the amount of Cre protein detected on the Western blots.

To further characterize the Cre-producing cell lines for both recombination and ability to support the growth of E1-deleted Adenovirus vectors, each cell line was separately infected with one of two different adenovirus vectors. The first viral vector was Ad β-gal, which carries a deletion of E1 and expresses β-galactosidase. This virus is insensitive to Cre recombinase and the level of β-galactosidase that it produces during infection reflects the infectivity of the cells and the amount of viral protein produced. The second viral vector was Adf*lox*β-gal, which carries a β-galactosidase gene flanked by identical *lox* sites. The expression of β-galactosidase from the second virus is sensitive to the level of Cre recombinase because Cre-mediated recombination will result in excision of the β-galactosidase gene. All of the Cre-expressing cell lines produced more intense X-gal staining with Ad β-gal than did the parental 293 cell line. All of the Cre-expressing cell lines produced about 5 times more intense staining when infected with Ad β-gal than with Adf*lox*β-gal. One cell line having the highest β-galactosidase expression with Ad β-gal, CRE8, was chosen for further experiments.

EXAMPLE 4. GROWTH OF HELPER RAD VECTOR Ψ5 ON CRE-EXPRESSING CELL LINE.

To confirm that the Ψ5 helper rAd is negatively selected on Cre-expressing cells, two types of experiments were carried out.

First, a mixture of Ψ5 and a similar Ad virus without *lox* sites, AdΔ, was used to infect either 293 cells or CRE8 cells. Virus from each infection was purified and DNA isolated. The DNAs were digested with BsaBI and separated on 0.6% agarose gels containing ethidium bromide. The ratio of left end fragments of 2.8 kb and 2.2 kb, for AdΔ and Ψ5 respectively, indicated the relative amount of each virus produced. For growth in 293 cells, the ratio was 60% Ψ5 to 40% AdΔ; for CRE8 cells, 5% Ψ5 and 95% AdΔ.

Second, the growth of Ψ5 in 293 cells or CRE8 cells was compared to the growth of Ad*lox*2 in the same cell lines. Ad*lox*2 is similar to Ψ5 but contains two non-equivalent *lox* sites rather than two equivalent *lox* sites. Equal viral yields were obtained in 293 cells, but the yield of Ψ5 in CRE8 cells was 1/20 of the yield of Ad*lox*2 in CRE8 cells.

EXAMPLE 5. CONSTRUCTION OF NEW RECOMBINANT ADENOVIRUSES WITH E1 SUBSTITUTIONS.

Several different replicating vectors were prepared from pAd*lox* by inserting one of several different genes into the expression cassette. Genes inserted included β-galactosidase, nitrous oxide synthase, T7 RNA polymerase, and nicotinic acetylcholine receptor α and β subunits. The replicating vectors were digested with SfiI to release the ITR bounded fragment and each digested replicating vector was mixed with Ψ5 DNA and used to transfect CRE8 cells. Plaques appeared after 3-4 days and viral lysates were prepared after 7 days. These lysates were used to infect CRE8 cells in a 10 cm dish. Packaged viral DNAs were prepared from the infected cells 2 days following infection. The DNAs were digested with BsaBI and the fragments separated on an agarose gel containing ethidium bromide. In each case, a new left end fragment appeared and the left end fragment corresponding to that from Ψ5 was not detected. The

recombinant viruses were also successfully tested for the expression of the substitute DNA.

EXAMPLE 6: PRODUCTION OF GUTLESS VIRUS

6μg each of SfiI digested ploxAβ and Ψ5 DNA's were co-transfected into a 6 cm dish of CRE8 cells. After three days the cells were overlaid with agar, and after seven days another layer of agar was added with 0.8 mg/ml of X-gal. The following day, 12 blue plaques were selected and freeze-thaw lysates made from the cells in the plaques. The loxAβ gutless virus was then amplified on CRE8 cells by successive passage of the virus onto 10^4, 10^5, 10^6 and finally 10^7 cells. At each step we added enough Ψ5 virus to insure that all of the cells were infected by the helper virus. Restriction analysis of packaged DNA from CRE8 cells infected with a portion of the virus from the 10^7 cell lysates showed that ten out of twelve contained an appreciable amount of loxAβ DNA (data for the first six are shown in **FIG. 5**). The amount of loxAβ DNA varied from a few percent to 20 percent of the total amount of virus. In two cases there was a large amount of helper virus DNA, indicating that Ψ5 had escaped selection. Two of the isolates were passaged further by infection into CRE8 cells without supplemental Ψ5 virus. The amount of loxAβ DNA never increased above 25 percent, and in both cases the helper viruses too escaped selection.

To assess the integrity of the loxAβ virus, fragments produced from a ClaI digestion of several of the isolates were analyzed (**FIG. 6**). ClaI digestion produces five major fragments from loxAβ, all of which migrated at their predicted sizes.

EXAMPLE 7: MUTATION OF Ψ5 TO ESCAPE SELECTION

In order to determine the nature of the Ψ5 virus after the transfection and amplification process, we plaque purified viruses from isolates 1 and 3 in **FIG. 5** taking eight plaques for each isolate from which we prepared DNA. Restriction analyses of these DNA's demonstrate that many contain deletions (**FIG. 7**). Taking representative viruses, we amplified two segments containing the loxP sites

by PCR and then sequenced the PCR products. The left loxP sites were intact. However, in all cases the right loxP site was missing.

To further analyze the stability of Ψ5 in CRE8 cells, we passaged Ψ5 virus through CRE8 cells. After eight passages, the virus began to grow well. Once again, we plaque purified isolates from the passaged virus and subjected the DNA to restriction analyses and sequencing. Out of ten viruses, one was still Ψ5, the rest had deletions of one or the other of the loxP sites.

EXAMPLE 8: PRODUCTION AND AMPLIFICATION OF LOXβA VIRUS IN 293 CELLS.

Only the final growth of gutless virus need be under negative selection by Cre recombinase in CRE8 cells. The plasmid ploxβA can be converted into a gutless virus by co-transfection into 293 cells. As this process is very inefficient, the virus must be amplified by some means. The method we have used is a positive selection based on an expressed β-galactosidase gene in the gutless virus. β-galactosidase has been chosen to demonstrate the concept. The selection could be for the gutless virus or against the helper virus or a complementation for viral growth of both (see FIG. 4). Ideally, unless the selection marker can be used therapeutically, it should be absent from the final therapeutic virus. This can be arranged by placing the marker between lox sites. These sites should be different from the lox site next to the packaging site so that they will not recombine. We have accomplished the selection by sorting on a FACS. The same result could be obtained by panning for an expressed extracellular membrane protein.

LoxβA virus was grown as follows. 6 μg each of ploxβA and Ψ5 DNA's were co-transfected into 293 cells. After seven days the cells were suspended in their media and a freeze thaw lysate was prepared. 1 ml of the lysate was used to infect $2x10^6$ 293 cells. At about 20 hours after infection the cells were removed from the dish by a brief treatment with trypsin, washed and loaded with florescein-digalactoside for one minute. The cells were then placed in chilled media and sorted on a FACstar cell sorter. Yield, 20,000 positive cells, which were then plated into 1 ml of tissue culture media and allowed to grow for 24 more hours. At twenty-four hours, the cells were suspended in their media and a freeze

thaw/lysate was prepared. This lysate was used undiluted, to infect 2.8 x10^6 more 293 cells. 60,000 β-galactosidase positive cells were recovered. A lysate was prepared from these cells which was used to infect 10^6 cells. These were sorted and 600,000 cells were recovered.

At this point 25% of the virus was used to infect 10^7 293 cells. We prepared packaged DNA from these cells, digested it with BglII and analyzed the fragments by gel electrophoresis. The total amount of loxβA virus was 5% of the total. In an attempt to change the ratio of Ψ5 to loxβA virus, we used small amounts of the virus to infect 293 cells then sorted the cells again. So we infected 2x10^6 293 cells with either 3, 6 or 12 μl of the virus and 0.67, 1.3 and 3.4 % of the cells were β-galactosidase positive, respectively. If the loxβA virus is as infectious as Ψ5, then most of the positive cells should have received one of each virus. We recovered 47,000 cells after infection with 12 μl of virus, from which we prepared a lysate. This was amplified by infecting 2x10^6 293 cells from which a lysate was prepared without sorting. Half of this lysate was used to infect 10^7 293 cells from which a 10 ml working stock of virus was made.

To determine the ratio of helper to gutless virus, and to see if the Cre mediated selection would still work, 1 ml of the working stock was used to infect 10^7 293 or CRE8 cells from which packaged DNA was prepared. The DNA's were digested with BglII and the fragments resolved by agarose gel electrophoresis (FIG. 5). From 293 cells, the loxβA was about 10% of the total viral DNA, and from CRE8 cells, the loxβA was about 50% of the DNA. This change results from a decrease in the amount of Ψ5 rather than an increase in the amount of loxβA. Clearly the Cre mediated selection was functioning as designed.

Next a 1::1 mixture of loxβA::Ψ5 viruses was prepared by infecting 10^7 CRE8 cells with 1 ml of the working stock yielding a 10 ml enriched stock. 1 ml of this virus was then used to infect either 10^7 293 or CRE8 cells from which packaged DNA was prepared. These DNA's were subjected to restriction analysis with BglII. The composition of the virus was 10% loxβA when grown in 293, and 50% loxβA when grown in CRE8 cells. These results show that in 293 cells the loxβA virus grows less well than the Ψ5 helper virus, and in CRE8 cells the viruses grow equally well.

5 ml of the enriched stock was used to infect $5x10^7$ CRE8 cells to produce 50 ml of viral lysate. 22.5 ml of this lysate was used to infect $2x10^8$ CRE8 cells. The resulting virus was purified by CsCl density gradient centrifugation. The purified virus was used to infect CRE8 cells at over a wide range of particles per cell. DNA was prepared from the packaged virus at each concentration, and restriction analysis was performed with BsaBI (FIG. 7). At this point, the ratio of loxβA to Ψ5 virus had improved to almost 90% loxβA virus, and this result was essentially independent of the amount of virus used for infection.

In an effort to assess the integrity of the loxβA virus, the samples were digested with a battery of restriction enzymes. In each case the terminal right end fragment was missing and unexpected bands were present. Except for the terminal right fragment, all of the predicted fragments were present for each enzyme used. The simplest explanation of these data is that the right end of the loxβA virus contains an insertion of a variable amount of DNA.

Next the viral DNA was treated with BstBI. BstBI does not cut the helper virus and should cleave once near the right end of loxβA. The digest produced a ladder of bands which represented right ends with 3 to 7 kb of extra DNA. These fragments were excised from the gel, treated with NaOH to remove the terminal protein, and ligated into the pSP73 cloning vector. Several clones were partially sequenced. The insertions came from either end of the helper virus. In a further analysis step, the virus was serially passaged with a mixture of insertions through four cycles of growth in CRE8 cells and then DNA was prepared for restriction analysis with BstBI. After five passages, the insertion size was clustered around 7 kb and the smaller insertions were gone. In an independent preparation of loxβA virus, insertions of around 7 kb were also observed.

In contrast to the data shown in FIG. 5, the loxβA viruses with insertions are able to grow about ten times better than the helper virus in CRE8 cells. As the insertions come from either end of the helper virus, and there appears to be a strong selection based on the size of the insertion, it seems unlikely that any specific information in the insertion is involved. Rather, the higher yield of loxβA virus with insertions results from increasing the loxβA chromosome to the optimal size for adenovirus. These viruses should preferably be within a lower size limit

of approximately 32 kb in order to maintain efficient packaging. In order to achieve efficient packaging of the recombinant virus, the minimum size is preferably within the range of 32 kb to 38 kb. The preferred minimum size of the virus is from 34 kb to 37 kb. Most preferred is the size of 35-36 kb. The optimum packaging size is that of the wild-type--roughly 36 kb. Recombinant Ad viruses that are smaller than 32 kb are not as stable, which creates significant problems for reliable delivery of the same recombinant vector for gene therapy. This discovery of the optimum packaging size is also important for manufacturing large quantities of the viral particles for commercial use, in which case packaging efficiencies on the order of wild type efficiencies are desired.

The fact that the same type of recombination event occurred both times we grew up the gutless virus further supports the finding of the minimum size for packaging efficiency and points to the insertion site as a hot spot for non-homologous recombination.

EXAMPLE 9: NEW HELPER VIRUSES

To lower the potential of homologous recombination, Ψ5 was modified by deleting the CMV promoter, multiple cloning site, and SV40 poly adenylation site from the virus. The new helper virus, called Ψ9, is identical to Ψ5 except the DNA between the first PvuII site (at position 454 in Ad5) and the Klenow treated ClaI site immediately before the second loxP site was removed. Additionally, a larger E3 deletion was incorporated (positions 28133 to 30818). The amount of Ψ9 DNA encapsidated in CRE8 compared to 293 cells with Ad β-gal as a standard was 1/6.

EXAMPLE 10: EFFECT OF PHASING OF LOXP SITES ON RECOMBINATION.

There are two versions of Ψ9, the one described above and one called Ψ9+17 with 17 bp of linker DNA inserted between the loxP sites. The center to center distance between the loxP sites is 301 bp for Ψ9 and 318 bp for Ψ9+17. Assuming 10.5 bp per helical turn, the sites are 28.66 and 30.28 turns apart, respectively. The relative encapsidation efficiencies were measured of these two viruses. Ψ9+17 was encapsidated 1/2 as well as Ψ9 (FIG. 11A and 11B).

These data indicate that phasing of the loxP sites is important for maximal recombination.

EXAMPLE 11: NEW PACKAGING SITES.

In the original definition of the Ad5 packaging site, two mutations which packaged at low efficiency were produced, A5 and B1. The B1 mutation (deletion of 271 to 356 in Ad5) was incorporated into the Ψ5 background, creating a virus called Ψ11. In a comparative growth assay, Ψ11 was mixed with Ad β-gal at 20 to 1 and infected 293 and CRE8 cells. Packaged DNA was prepared from each cell type and a PCR analysis was performed on the DNA's (**FIG. 12**). For growth on 293 cells, Ψ11 is about 1/80 of Ad β-gal, in agreement with published estimates. For growth in CRE8 cells, Ψ11 was undetectable.

The packaging site from Ad5 has varied degree of relationship to the same region from other serotypes of adenovirus.. Substitutions were prepared of the Ad5 packaging domain (194 to 454 in Ad5) with the equivalent regions from Ad7 (203 to 466), Ad12 (101 to 401) and Ad40 (101 to 374), and these substitutions were built into a Ψ9 background. The Ad7 substitution was viable, creating Ψ7. In a measurement of the relative encapsidation efficiency, Ψ7 was encapsidated at 30 and 5.9% of a similar virus with a normal Ad5 packaging site when grown in 293 and CRE8 cells respectively (**FIG. 13**).

EXAMPLE 12. PURIFICATION OF GUTLESS VIRUS BY SUCCESSIVE PASSAGE THROUGH CRE8.

10^8 CRE8 host cell are infected with a 1::1 mixture of loxβA and ψ5 at a multiplicity of infection of 5 for the ψ5. The yield was 4 x 10^{12} particles at 85% loxβA and 15% ψ5. This virus mixture was used to infect 10^8 CRE8 cells at a multiplicity of 5 for the ψ5. The yield of virus was 4 x 10^{11} and the amount of ψ5 was about 3%.

Example 13. Constructing New Recombinant Adenoviruses with Substitutions

As shown in **FIG. 14**, new recombinant adenoviruses with substitutions in the E1 region can be generated using $\psi 5$ as a helper vector that supplies an adenoviral backbone, a replicating vector with a single recombination site, a loxP site, and a cre-expressing, E1A+, E1B+ host cell line. The cre recombinase catalyzes recombination between $\psi 5$ and the replicating vector with a single loxP site, providing an efficient means to construct recombinant adenoviruses with substitute DNA in place of the E1 region.

The Cre-*lox* recombination technique for generating new recombinant adenovirus vectors with substitutions was compared to a popular overlap recombination method for production of recombinants. A series of recombinations between transfected adenovirus donor DNAs (helper rAd vectors) and replicating vectors and then prepared lysates 3, 7, 10, and 14 days after transfection and screened for the presence of *lacZ*-positive virus, using the FDG assay. These recombinations were used to examine the effects of two factors, the source of the donor DNA and the mechanism of recombination. First, either Ψ5 viral DNA or pBHG10 plasmid DNA was transfected along with replicating vectors marked with *lacZ* genes. Second, Ψ5 DNA was combined with *lacZ*-marked replicating vectors having either a *loxP* site for Cre-*lox* recombination or a 5.5-kb adenovirus fragment for overlap recombination. Recombination into Ψ5 viral DNA by either method produced by *lacZ* virus by 4 days, with Cre-*lox* recombination being slightly more efficient. In contrast, overlap recombination into PBHG10 plasmid DNA required 10 days before lacZ virus appeared, and then it did so only when the linear replicating vector was used.

An important factor in the recombination process might be the topology of the replicating vector plasmid. To test this, pAdlox plasmids carring a β-galactosidase marker gene (pAdlox β-gal) either uncut or treated in various ways was transfected along with Ψ5 viral DNA. After 3 days lysates were prepared and titered for β-galactosidase-positive virus by the FDG assay. All of the transfections produced recombinant virus but with a substantial variation in efficiency depending on the treatment of the plasmid. Cutting the pAdlox plasmid at both ITRs (*SfiI*) or at both ITRs plus cutting off the right ITR (*Sfi*I) or at both ITRs plus cutting off the right ITR (*Sfi*I plus *Apa*I) produced equally high yields of

recombinant virus. Cutting the plasmid with *Sca*I such that the ITRs remained buried in the plasmid sequences reduced the yield to 17% of *Sfi*I-cut plasmid, and circular plasmid produced the least amount of recombinant virus, at about 4% of the rate of plasmid cleaved at the ITRs.

Next, transfection was used to introduce the replicating vector plasmid and compared infection with transfection to introduce Ψ5 DNA. Cells were transfected with the replicating vector and infected with Ψ5 virus at various times relative to the infection. One sample was cotransfected as before. Virus produced from each sample was used to infect CRE8 cells. A restriction analysis was performed on the packaged DNA to monitor the ratios of the helper rAd vector and recombinant viruses. Contransfection of Ψ5 and pAdlox β-gal produced a virtually helper-free stock of recombinant virus in 10 days. In contrast, infection of the helper rAd virus combined with transfection of the replicating vector plasmid produced less recombinant virus and a significant amount of helper contamination in a similar time frame.

In the foregoing experiment, there was very little rAd helper virus DNA in cotransfected samples after the second passage through CRE8 cells. To determine more precisely how effectively the helper virus was removed during successive passages through CRE8 cells, we took the virus mixture from the cotransfection and passed it two more times through CRE8 cells, making lysates at each passage. Lysates which were stained with X-Gal and neutral red to distinguish the recombinant virus plaques from Ψ5 or mutant viruses were then diluted and infected into 293 cells for plaque analyses. The initial transfection (passage 1) contained 30% Ψ5 virus. One passage through CRE8 cells reduced the Ψ5 virus to 3%. A further passage through CRE8 cells reduced the concentration of donor virus to 0.2%. See Hardy et al., J. Virology, 71(3):1842-1849 (1997) (incorporated herein by reference).

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A recombinant adenovirus vector comprising:

(a) a left inverted terminal repeat;

(b) one or more different recombination sites;

(c) an adenovirus packaging site;

(d) a foreign DNA sequence; and

(e) a right inverted terminal repeat;

wherein said recombinant adenovirus vector is incapable of expressing any adenovirus genes.

2. The vector of claim 1, wherein said vector contains no more than 600 basepairs of adenovirus sequence.

3. A therapeutic recombinant adenovirus vector comprising:

(a) a foreign DNA sequence, wherein said foreign DNA sequence comprises a sequence encoding a therapeutically effective polypeptide; and

(b) adenovirus DNA consisting essentially of left and right inverted terminal repeats, an adenovirus packaging site, and one or more different recombination sites, wherein said vector ranges in size from 32 kb to 38 kb.

4. The vector of claim 3, wherein said vector ranges in size from 34 to 37 kb.

5. The vector of claim 4, wherein said vector ranges in size from 35 to 36 kb.

6. The vector of claim 3, wherein said foreign DNA sequence ranges in size from 31.4 to 37.4 kb.

7. The vector of claim 3, wherein said adenovirus DNA sequence contains a lox site.

8. A recombinant adenovirus helper vector comprising:

(a) left and right inverted terminal repeats;

(b) one or more adenovirus genes necessary for either replication or packaging of adenovirus vectors; and

(c) a packaging site flanked by at least a first pair of identical recombination sites.

9. The helper vector of claim 8, wherein said identical recombination sites are in phase with each other.

10. The helper vector of claim 8, wherein said first pair of identical recombination sites is flanked by a second pair of identical recombination sites, wherein said recombination sites of said first and second pairs are different.

11. The helper vector of claim 10, wherein said first pair of identical recombination sites are loxP sites and said second pair of identical recombination sites are lox511 sites.

12. The helper vector of claim 10, further comprising a third set of identical recombination sites different from said first and second sets, wherein said second set is flanked by said third set.

13. The helper vector of claim 10, wherein said identical recombination sites are in phase with each other.

14. The helper vector of claim 8, wherein said packaging site is a synthetic packaging site.

15. The helper vector of claim 8, wherein said packaging site is from an adenovirus serotype different from the adenovirus serotype from which the one or more adenovirus genes are derived.

16. The helper vector of claim 8, wherein said packaging site is no more than 600 base pairs from the left ITR or from the right ITR.

17. A method of preparing a therapeutic recombinant adenovirus comprising:

(a) obtaining a therapeutic recombinant adenovirus vector comprising a foreign DNA sequence and adenovirus DNA sequences consisting essentially of left and right inverted terminal repeats, an adenovirus packaging site, and one or more different recombination sites;

(b) obtaining a recombinant adenovirus helper vector comprising left and right inverted terminal repeats, one or more adenovirus genes encoding proteins necessary for either replication or packaging of adenovirus vectors, and a packaging site flanked by at least a first pair of identical recombination sites in direct orientation with respect to one another, wherein for each pair of identical recombination sites present in said helper vector there is one identical recombination site in said therapeutic recombinant adenovirus vector,

(c) transfecting a eukaryotic host cell susceptible to adenovirus infection with said therapeutic recombinant adenovirus vector and said recombinant adenovirus helper vector;

(d) isolating packaged viral particles; and

(e) infecting a eukaryotic host cell with said isolated packaged viral particles, wherein said eukaryotic host cell is susceptible to adenovirus infection and is capable of expressing a recombinase capable of mediating recombination between said recombination sites.

18. The method of claim 17, wherein the polypeptides encoded by said helper vector are capable of recognizing the ITRs of the helper vector and the ITRs and packaging site of the therapeutic rAd vector.

19. The method of claim 17, wherein said recombination sites are loxP sites and said recombinase is Cre.

20. The method of claim 17, wherein said host cell is capable of expressing Cre in an intracellular concentration of between 0.01 μM and 50 μM.

21. The method of claim 17, wherein steps (d) and (e) are repeated once.

22. The method of claim 17, wherein steps (d) and (e) are repeated twice.

23. A system for preparing therapeutic recombinant adenovirus vector particles comprising:

(a) a therapeutic recombinant adenovirus vector comprising a foreign DNA sequence, wherein said foreign DNA sequence comprises a sequence encoding a therapeutically effective polypeptide, and adenovirus DNA consisting essentially of left and right inverted terminal repeats, an adenovirus packaging site, and one or more different recombination sites, wherein said vector ranges in size from 32 kb to 38 kb;

(b) a recombinant adenovirus helper vector comprising left and right inverted terminal repeats, and a packaging site flanked by at least a first pair of identical recombination sites;

(c) a eukaryotic host cell susceptible to adenovirus infection, wherein said host cell is capable of expressing of a recombinase capable of mediating recombination between said recombination sites;

wherein for each pair of identical recombination sites in said helper vector there is an identical recombination site in said therapeutic recombinant adenovirus vector.

24. The system of claim 23, wherein said first pair of identical recombination sites in said helper vector are flanked by a second pair of identical recombination sites, and said recombination sites of said first and second pairs are different.

25. The system of claim 24, wherein one pair of recombination sites are loxP and another pair of recombination sites are lox511.

26. The system of claim 23, wherein said foreign DNA sequence ranges in size from 36 kb to 37.4 kb.

27. The system of claim 23, wherein at least one pair of recombination sites are in phase with each other.

28. A substantially pure preparation of packaged therapeutic recombinant adenovirus vector particles, wherein said particles are prepared by the method of claim 16.

29. A composition for treating a genetic disorder comprising a therapeutically effective amount of a substantially pure preparation of packaged therapeutic recombinant adenovirus vector particles, wherein said particles comprise the vector of claim 1.

30. A method of treating a mammal having a genetic disorder, comprising administering the composition of claim 29.

31. A method of generating new recombinant adenovirus vectors with substitutions comprising:

(a) providing a helper vector of claim 8,

(b) providing a replicating vector comprising an adenovirus packaging site flanked by adenovirus ITRs, a recombination site, substitute DNA inserted between said packaging site and said recombination site, wherein said recombination site is identical to said first pair of recombination sites in said helper vector;

(c) transfecting recombinase-expressing host cells with said helper vector and said replicating vector;

(d) isolating packaged viral particles; and

(e) infecting recombinase-expressing host cells with said isolated packaged viral particles.

32. A method of generating new recombinant adenovirus vectors with substitutions comprising:

(a) providing a helper vector of claim 8,

(b) providing a non-replicating vector comprising an adenovirus ITR, an adenovirus packaging site, a substitute DNA sequence, and a recombination site,

wherein said recombination site is identical to said first pair of recombination sites in said helper vector;

(c) transfecting recombinase-expressing host cells with said helper vector and said replicating vector;

(d) isolating packaged viral particles; and

(e) infecting recombinase-expressing host cells with said isolated packaged viral particles.

33. A method of generating new recombinant adenovirus vectors with substitutions comprising:

(a) providing a helper vector of claim 8,

(b) providing a replicating vector comprising an adenovirus packaging site flanked by adenovirus ITRs, a recombination site, substitute DNA inserted between said packaging site and said recombination site, wherein said recombination site is identical to said first pair of recombination sites in said helper vector;

(c) transfecting recombinase-expressing host cells with said replicating vector and infecting with a helper virus derived from said helper vector;

(d) isolating packaged viral particles; and

(e) infecting recombinase-expressing host cells with said isolated packaged viral particles.

34. The method of claim 31, wherein said substitutions are in the E1 region, said recombinase-expressing host cells are cre-expressing host cells comprising E1A+ and E1B+ adenovirus genes, and said recombination sites are lox sites.

35. A system for generating new recombinant adenovirus vectors with substitutions comprising:

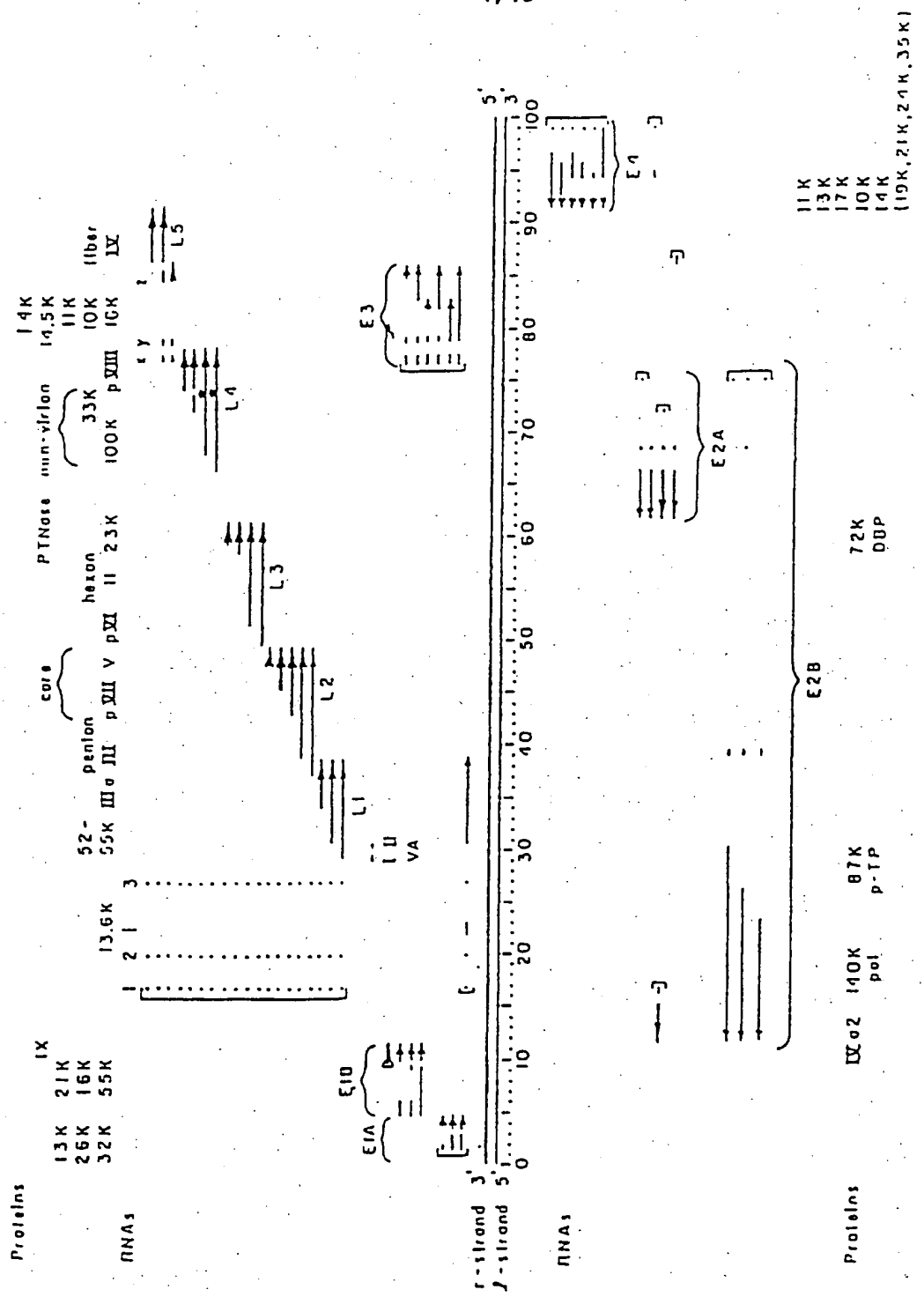
(a) a helper vector of claim 8,

(b) a replicating vector comprising an adenovirus packaging site flanked by adenovirus ITRs, a recombination site, substitute DNA inserted between said

packaging site and said recombination site, wherein said recombination site is identical to said first pair of recombination sites in said helper vector; and

(c) a recombinase-expressing host cell line susceptible to adenovirus infection.

5


Proteins
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13K 21K
26K 16K
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V
pVII
hexon
II 23K
PTNase
non-virion
33K
100K
pIX
pVIII
14K
14.5K
11K
10K
16K
fiber
IX
L1
L2
L3
L4
L5
I II
VA
E1A
E1B
E3
E4
E2A
E2B
r-strand
l-strand
0 10 20 30 40 50 60 70 80 90 100
11K
13K
17K
10K
14K
(19K,21K,24K,35K)
72K
DBP
IVa2
140K
pol
87K
p-TP
Proteins

FIGURE 1

LOX SITES

lox P	(SEQ ID NO:1)	ATAACTTCGTATA ATGTATGC TATACGAAGTTAT
lox 511	(SEQ ID NO:2)	ATAACTTCGTATA ATGTATAC TATACGAAGTTAT
lox 514	(SEQ ID NO:3)	ATAACTTCGTATA ATGTACGC TATACGAAGTTAT
lox Psym	(SEQ ID NO:4)	ATAACTTCGTATA ATGTACAT TATACGAAGTTAT

FIGURE 2

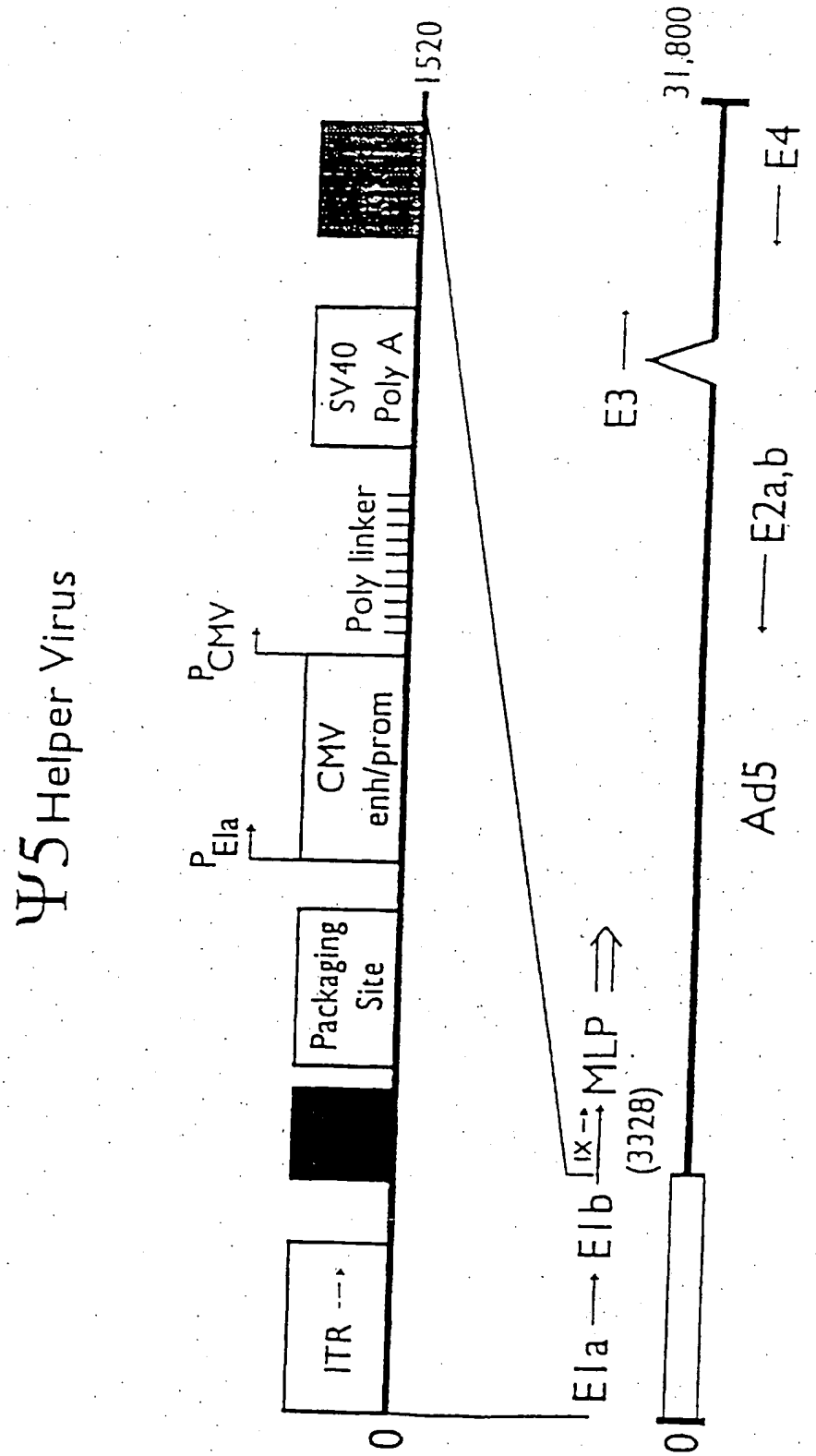

Ψ5 Helper Virus
ITR
Packaging Site
CMV enh/prom
P_{Ela}
P_{CMV}
Poly linker
SV40 Poly A
1520
0
E1a
E1b
IX
MLP
(3328)
E3
E4
E2a,b
Ad5
31,800
0

FIGURE 3

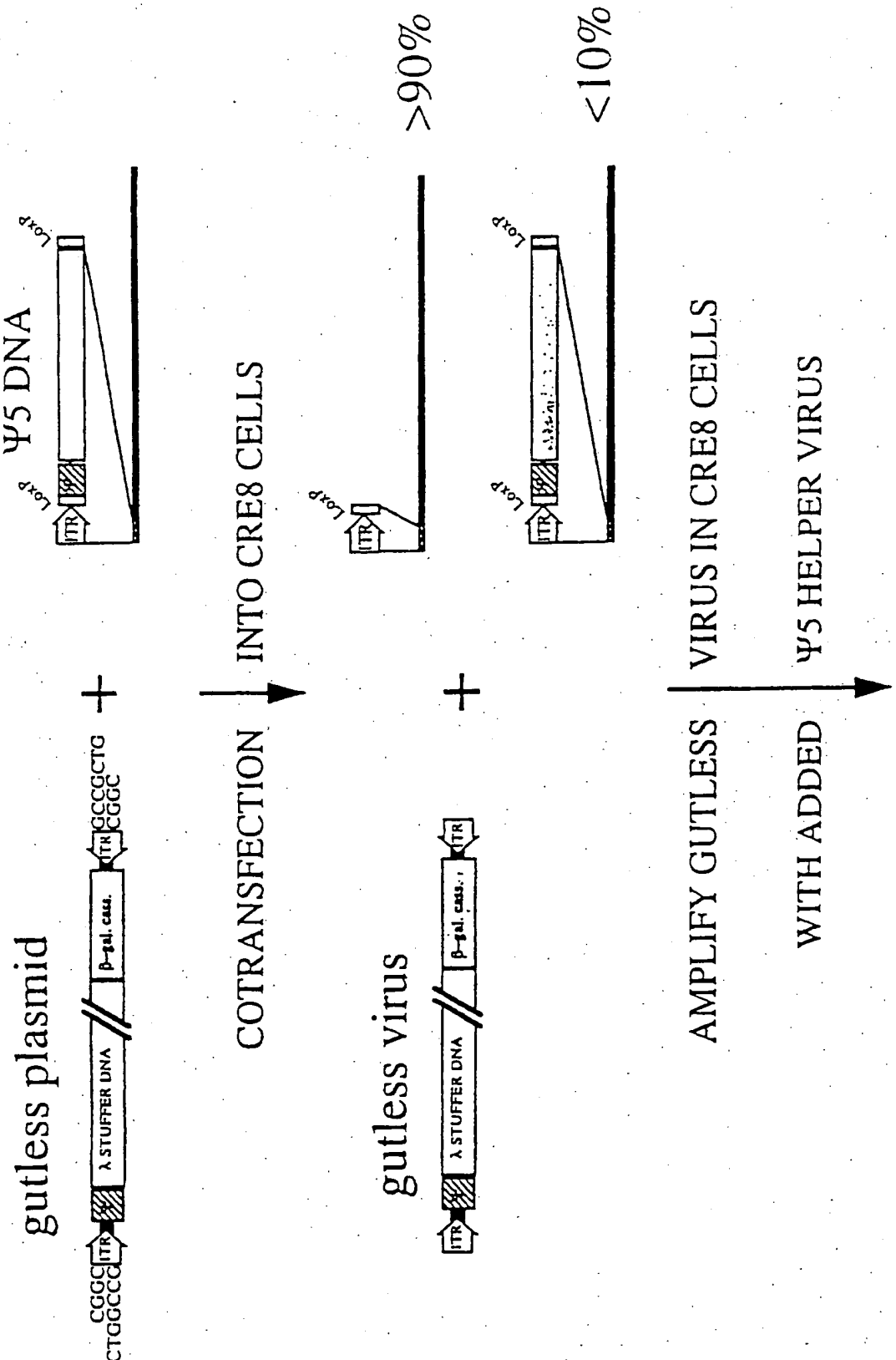

gutless plasmid
CGGC
CTGGCCCC
ITR
λ STUFFER DNA
β-gal. exp.
ITR
GCCGCTG
CGGC
+
COTRANSFECTION
INTO CRE8 CELLS
Ψ5 DNA
LoxP
ITR
LoxP
>90%
<10%
gutless virus
ITR
λ STUFFER DNA
β-gal. exp.
ITR
+
AMPLIFY GUTLESS
VIRUS IN CRE8 CELLS
WITH ADDED
Ψ5 HELPER VIRUS

FIG. 4.

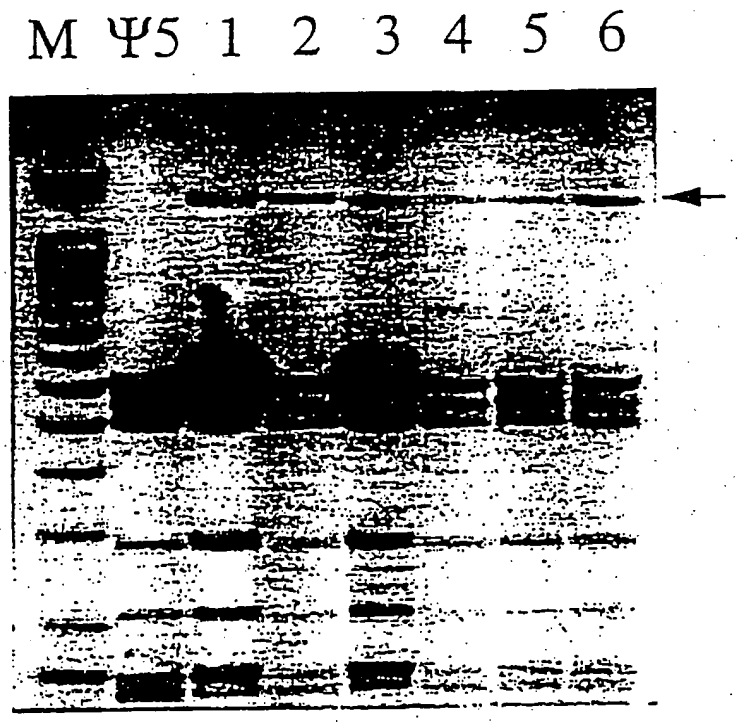


FIG. 5.

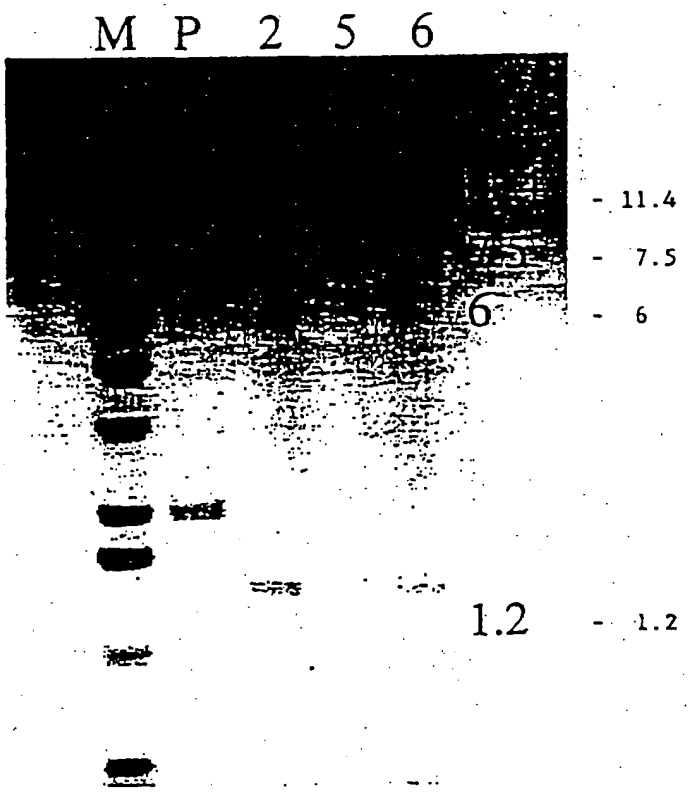


FIG. 6.

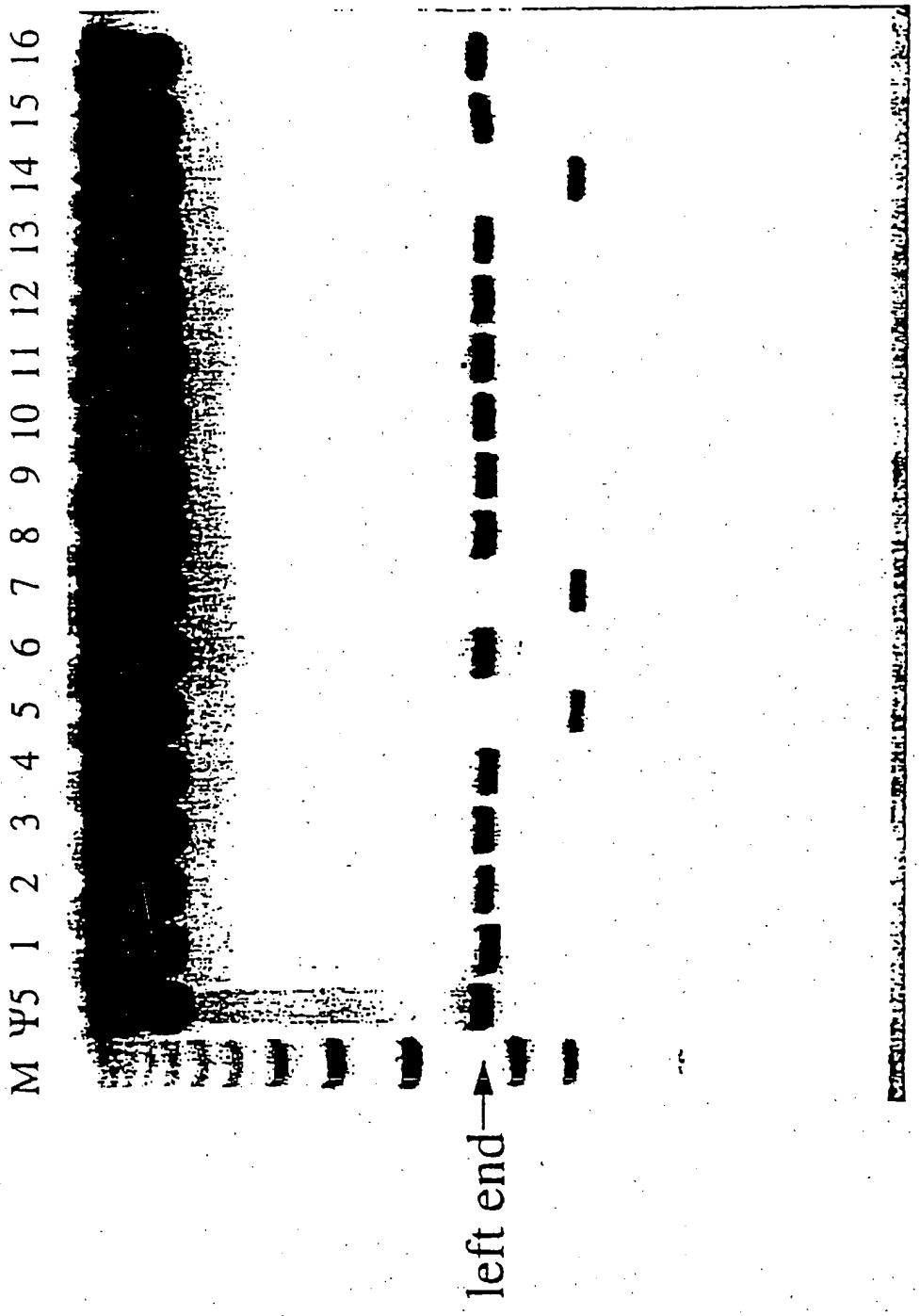


FIG. 7.

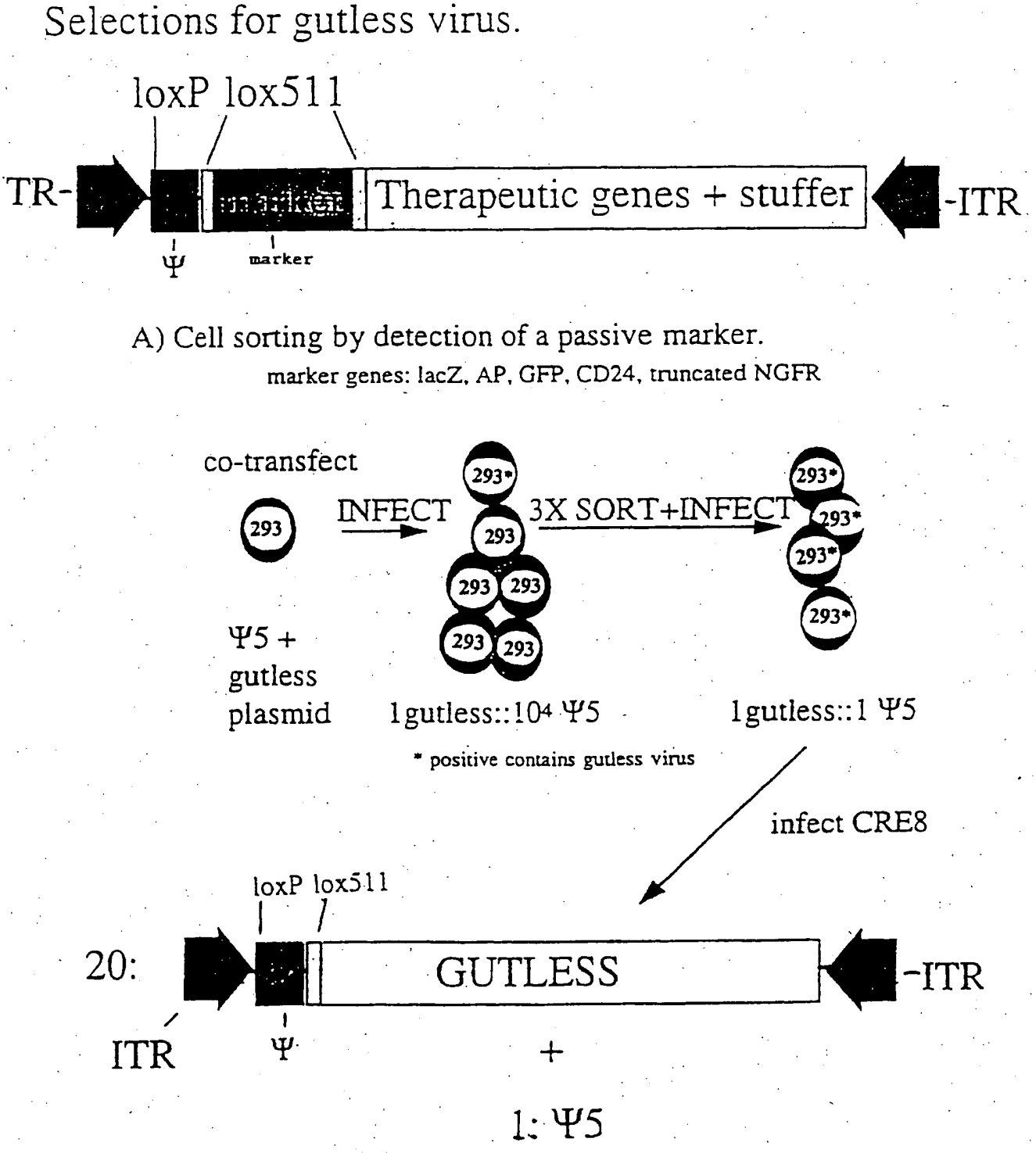

Selections for gutless virus.
loxP lox511
TR-
Therapeutic genes + stuffer
-ITR
Ψ
marker
A) Cell sorting by detection of a passive marker.
marker genes: lacZ, AP, GFP, CD24, truncated NGFR
co-transfect
293
INFECT
293*
293
293 293
293 293
3X SORT+INFECT
293*
293*
293*
293*
Ψ5 +
gutless
plasmid
1gutless::10⁴ Ψ5
1gutless::1 Ψ5
* positive contains gutless virus
infect CRE8
loxP lox511
20:
GUTLESS
-ITR
ITR
Ψ
+
1: Ψ5

FIG. 8A.

B) COMPLEMENTATION OF A VIRAL FUNCTION SUCH AS E4 OR VAI

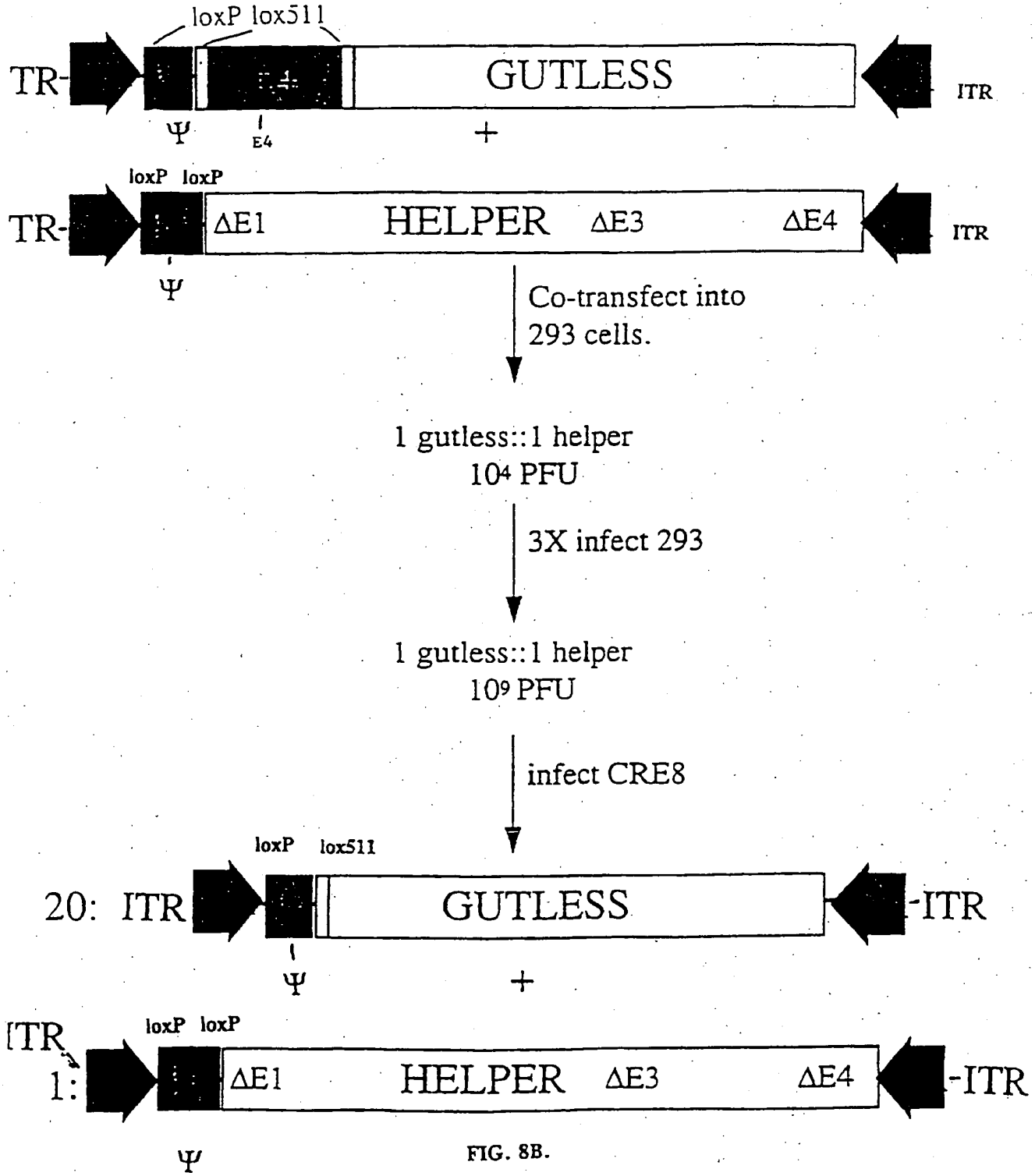


FIG. 8B.

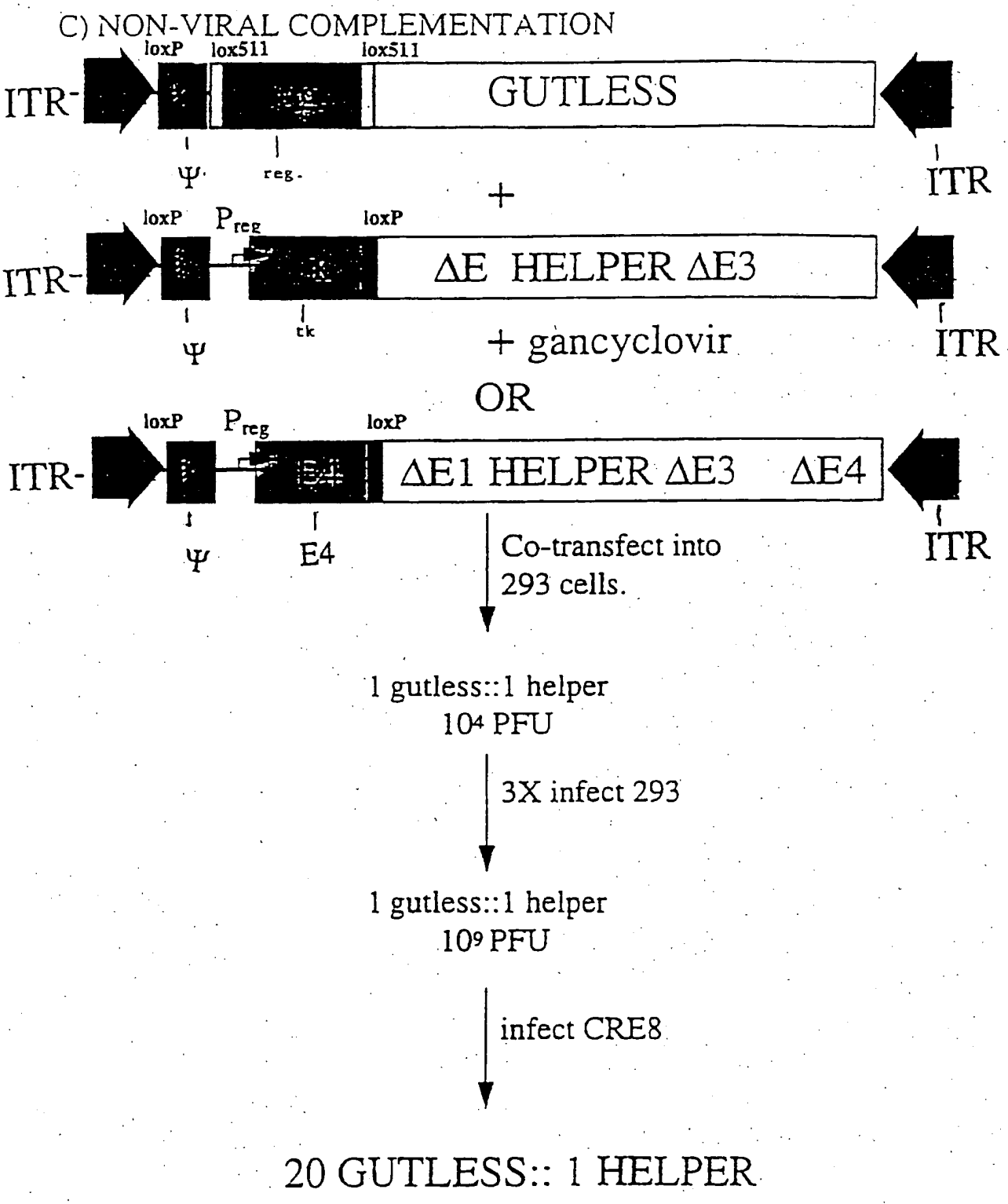

C) NON-VIRAL COMPLEMENTATION
loxP
lox511
lox511
ITR-
GUTLESS
Ψ
reg.
ITR
+
loxP
Preg
loxP
ITR-
ΔE HELPER ΔE3
Ψ
tk
ITR
+ gancyclovir
OR
loxP
Preg
loxP
ITR-
ΔE1 HELPER ΔE3 ΔE4
Ψ
E4
ITR
Co-transfect into
293 cells.
1 gutless::1 helper
10⁴ PFU
3X infect 293
1 gutless::1 helper
10⁹ PFU
infect CRE8
20 GUTLESS:: 1 HELPER

FIG. 8C.

GUTLESS VIRUS ENRICHMENT BY GROWTH IN CRE8 CELLS

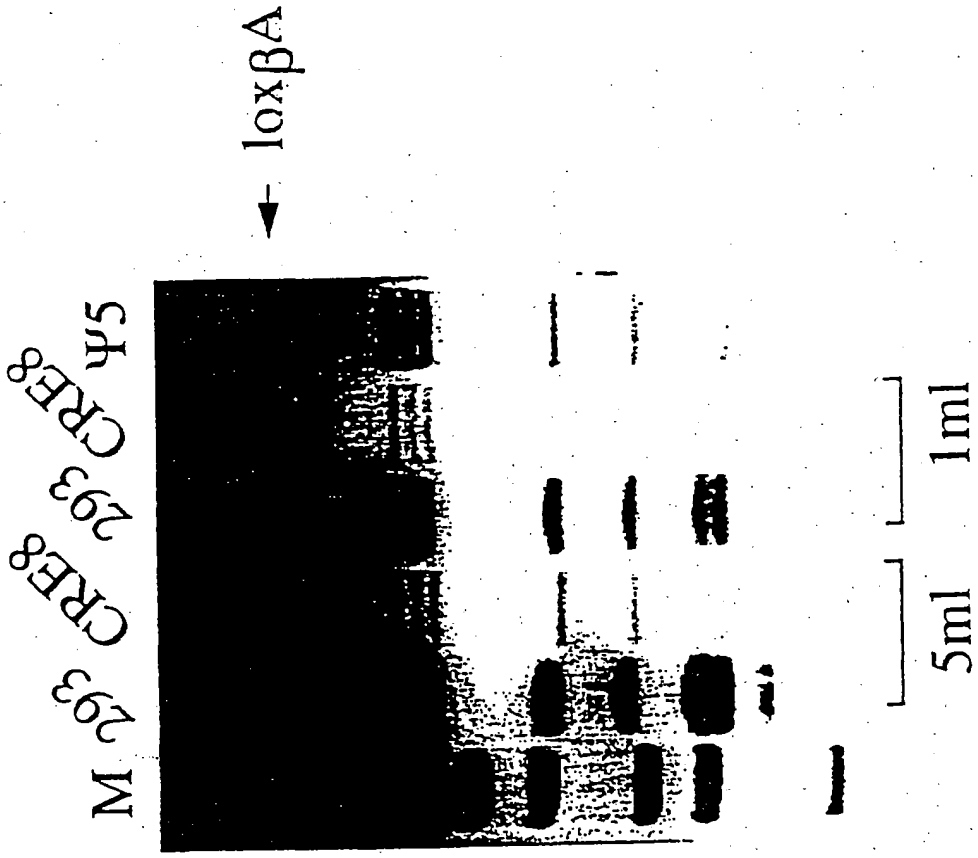


FIG. 9.

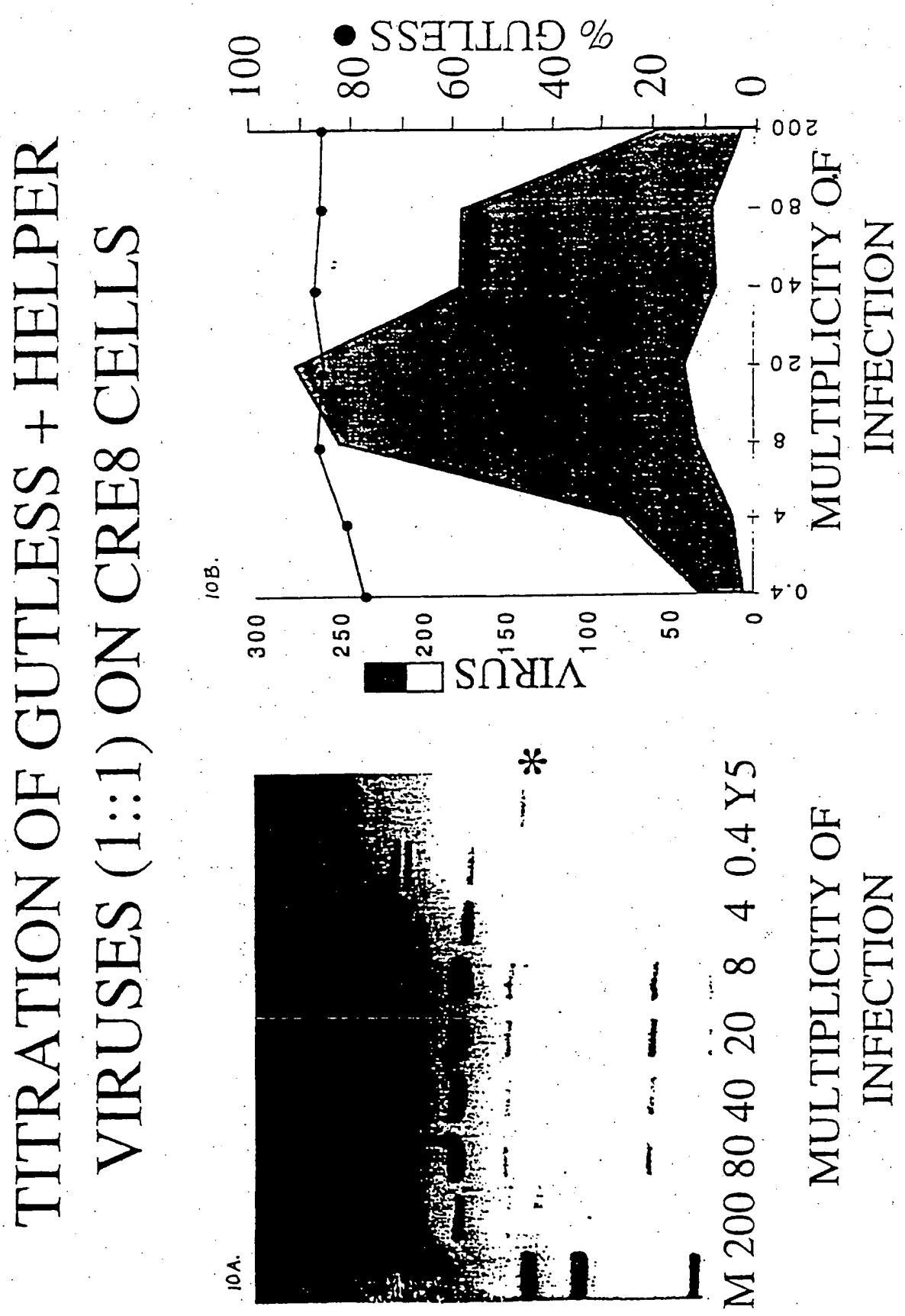
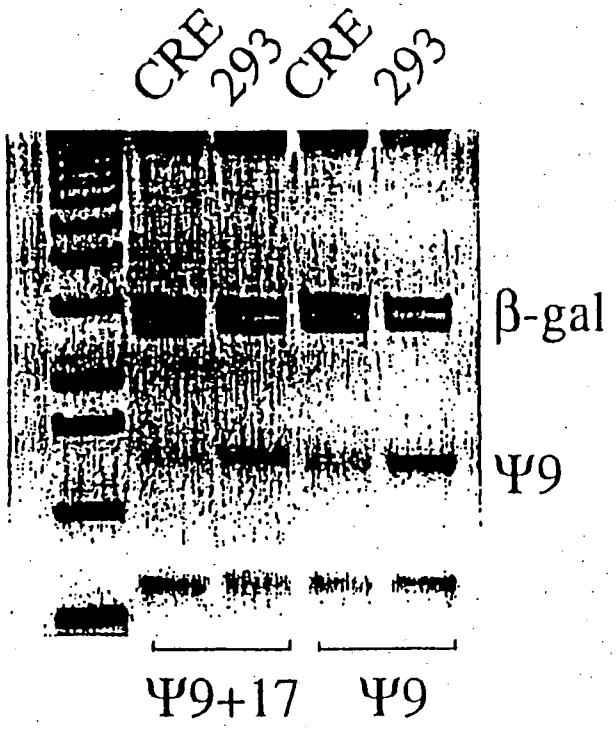
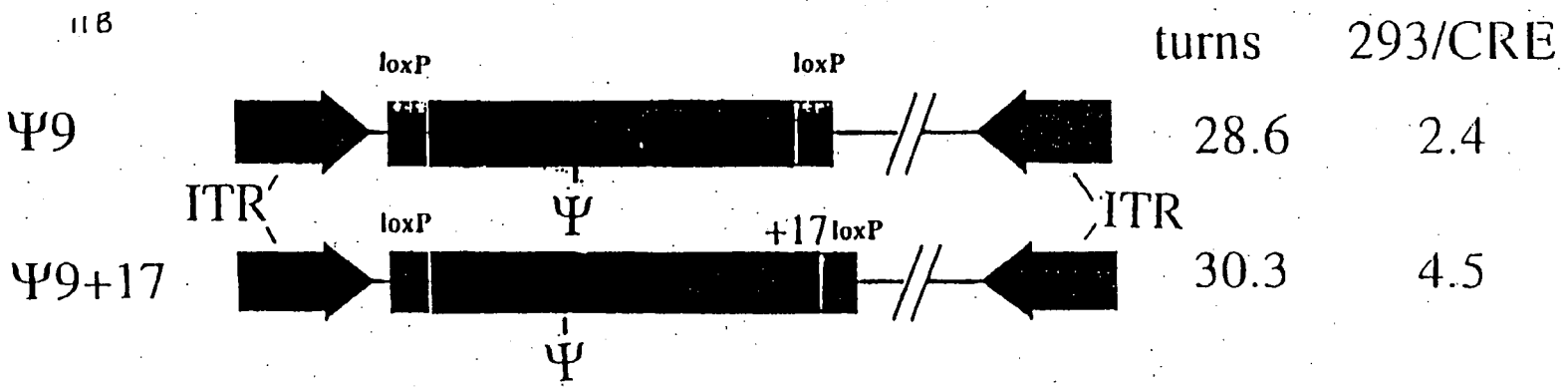

TITRATION OF GUTLESS + HELPER VIRUSES (1::1) ON CRE8 CELLS
10A.
M 200 80 40 20 8 4 0.4 Y5
MULTIPLICITY OF INFECTION
*
10B.
VIRUS
% GUTLESS
MULTIPLICITY OF INFECTION

FIG. 10.

PHASING IN CRE/LOX RECOMBINATION



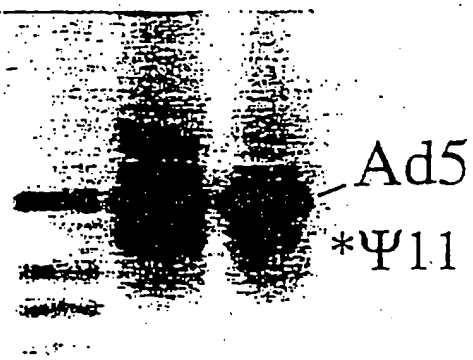


	turns	293/CRE
Ψ9	28.6	2.4
Ψ9+17	30.3	4.5

FIG. 11.

Ψ11 PACKAGING IN 293 AND CRE8 CELLS

12A.



M 293 CRE8

12B.

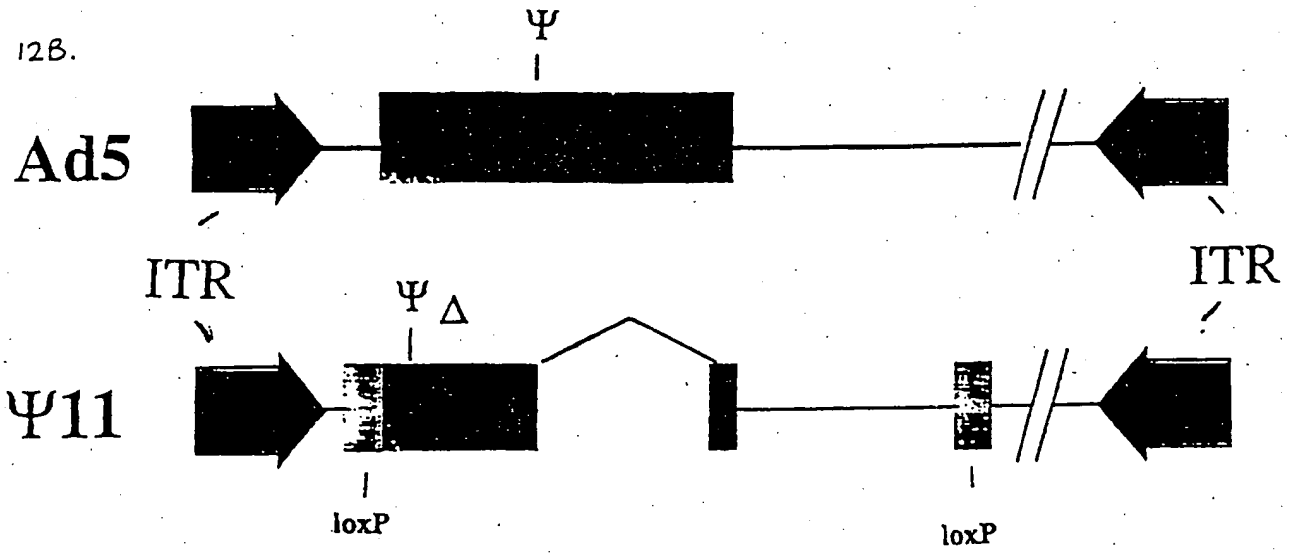
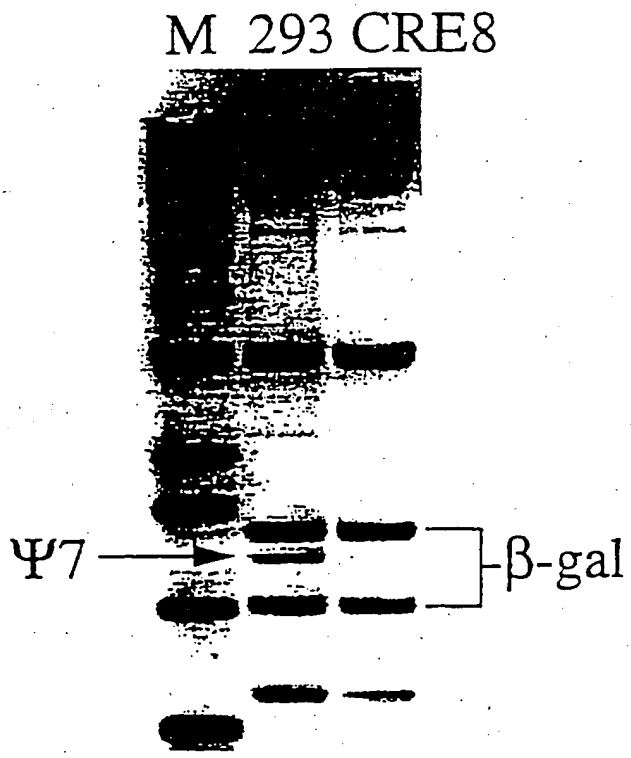


FIG. 12.

Encapsidation Efficiency of Ψ7 in 293 and CRE8 Cells

13A.



13B.

	293	CRE8
β-gal	100	100
Ψ7	31	5.9

FIG. 13.

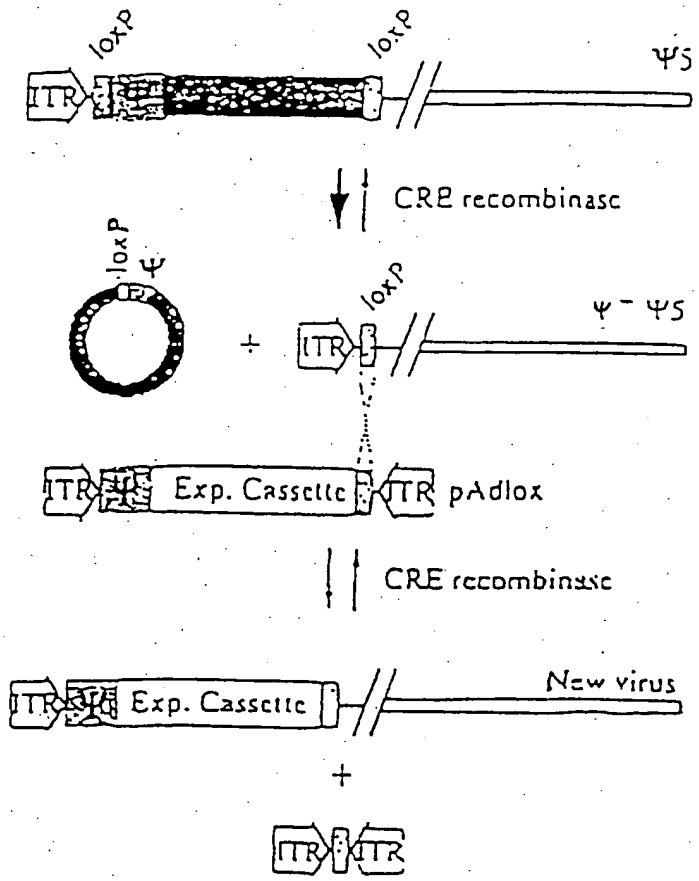

loxP
loxP
Ψ5
ITR
CRE recombinase
loxP
Ψ
loxP
Ψ - Ψ5
ITR
ITR
Exp. Cassette
ITR
pAdlox
CRE recombinase
ITR
Exp. Cassette
New virus
+
ITR
ITR

FIG. 14

JOURNAL OF VIROLOGY, May 1990, p. 2047–2056
0022-538X/90/052047-10$02.00/0

Vol. 64, No. 5

Adenovirus Type 5 Packaging Domain Is Composed of a Repeated Element That Is Functionally Redundant

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Received 1 November 1989/Accepted 23 January 1990

Previous analyses have demonstrated that adenovirus DNA is packaged into virions in vivo in a polar, left-to-right fashion. The packaging of viral DNA is dependent on *cis*-acting elements at the left end of the genome. In this report, we describe a genetic analysis of the sequences that are required for efficient packaging of adenovirus type 5 (Ad5) DNA. Our results demonstrate that the Ad5 packaging domain (nucleotides 194 to 358) is composed of at least five distinct elements that are functionally redundant. An AT-rich repeated sequence motif, the A repeat, is located in four of five of these regions; the fifth region is also AT rich. The efficiency of viral packaging depends on the number of individual A repeats that are present in the viral genome. The deletion of the entire packaging domain resulted in the loss of virus viability. A virus that contains a multimerized oligonucleotide corresponding to A repeat II in place of the packaging domain could package viral DNA, although with reduced efficiency compared with that of the wild-type virus. Our results also suggest that the spacing of specific sequences at the left end of the Ad5 genome are important for enhancer region function in vivo.

The selective packaging of adenovirus DNA into virions late in infection appears to involve the specific recognition of viral DNA sequences and then condensation and encapsidation of the viral genome into preformed capsids (4, 6, 7, 10, 20, 36). Incomplete particles of adenoviruses in groups A, B, and C contain subgenomic segments of the viral genome that are enriched for left-end sequences, suggesting that the viral genome is packaged in a polar fashion from left to right in a manner that is dependent on a *cis*-acting element(s) (5, 14, 15, 37). Evidence to support this idea has been obtained from the analysis of evolutionary variants of adenovirus types 3 and 16 (Ad3 and Ad16, respectively). With Ad3, the analysis of a group of mutants that contain rearrangments at the left end of the viral genome indicates that the sequences that specify polar packaging lie between nucleotides (nt) 319 and 390 (29). With Ad16, the analysis of variants that contain different amounts of left-end sequences duplicated at the right end of the genome has shown that the Ad16 sequences between nt 290 and 390 are required for efficient polar packaging (14). Because of a *cis* requirement for virus replication, it has not been possible to determine whether sequences within the viral inverted terminal repeat (Fig. 1A) (approximately 100 to 150 nt at each end of the adenovirus genome, depending on the virus type) are also involved in the packaging process.

We previously identified a *cis*-acting packaging domain that is located between nt 194 and 358 at the left end of the Ad5 genome (Fig. 1A) (16, 17). Mutant viruses that lack this region are nonviable, but this defect is complemented by the insertion of the left terminal 355 nt at the right end of the Ad5 genome (17). The Ad5 packaging domain functions at either end of the viral genome, in an inverted orientation, and can be moved within several hundred base pairs of its original location without a reduction in virus yield (16). The Ad5 packaging domain, therefore, shares properties with eucaryotic transcriptional enhancer elements. A repeated sequence motif, the A repeat, is present in the Ad5 packaging domain and has been implicated in the packaging process (Fig. 1B, AI through AV) (18).

The Ad5 packaging domain is located within the early region 1A (E1A) enhancer region (Fig. 1 and 2) (17, 18). Two distinct enhancer elements in this region that regulate viral gene expression have been defined. Enhancer element I is a repeated sequence that specifically regulates E1A transcription (17, 18); a cellular protein that binds to this motif has been identified (3). Viral element I mutants are efficiently complemented when the E1A products are provided in *trans*. Enhancer element II spans a region of approximately 30 base pairs (bp) (nt 250 to 280) and enhances transcription from each of the viral early transcription units (18). The reduced expression of the early gene products with viral element II mutants results in decreased viral DNA replication and a three- to sevenfold reduction in virus yield compared with that of the wild-type virus (16, 18). The *cis*-acting defect in virus yield with element II mutants is complemented in *trans* in a mixed infection with a wild-type virus in which the wild-type virus provides early proteins required for efficient replication (16, 18).

In this report, we describe a genetic analysis of the sequences that are required for the efficient packaging of Ad5 DNA. Our results demonstrate that the Ad5 packaging domain (nt 194 to 358) is composed of multiple, functionally redundant elements. At least five distinct regions in the Ad5 packaging domain are involved in the packaging process. Four of these regions contain a repeated sequence motif (the A repeat, 5′-$\frac{A}{T}$AN$\frac{A}{T}$TTTG-3′) (Fig. 2). The efficiency of viral packaging depends on the number of A repeats in the viral genome. A virus that contains a multimerized oligonucleotide of a single A repeat in place of the entire packaging domain could package viral DNA, albeit with reduced efficiency compared to that of the wild-type virus. Finally, we demonstrate that the phenotype of viral enhancer element II

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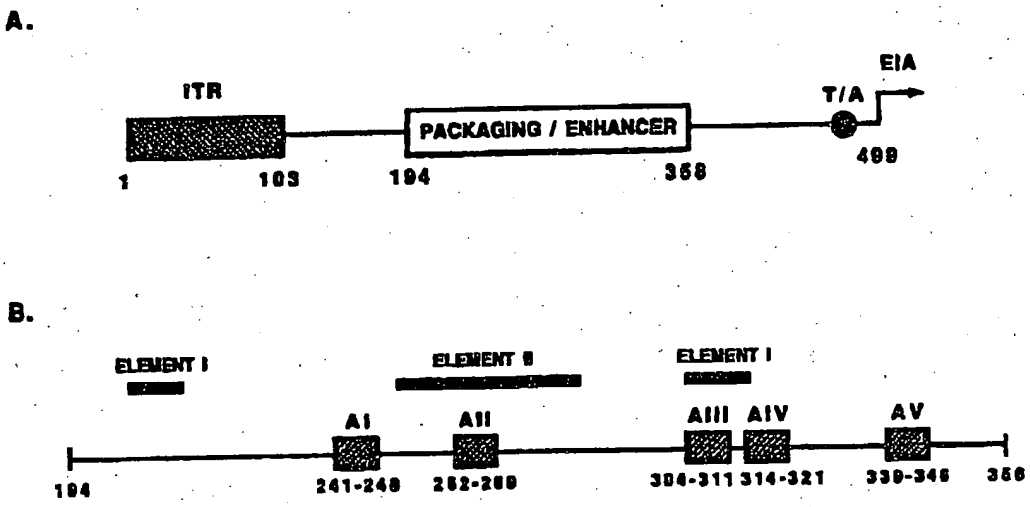

FIG. 1. (A) Schematic diagram of the left end of the Ad5 genome including the E1A 5' flanking region. Nucleotide numbers relative to the left terminus of the genome are indicated below the line. The inverted terminal repeat (ITR) and the packaging-enhancer region are represented by hatched and open boxes, respectively. The TATA box motif (T/A) is indicated by the stippled circle. The E1A start site (nt 499) and transcription unit are indicated by the arrow. (B) Schematic view of the packaging-enhancer region (nt 194 to 358). The positions of A repeats I through V are shown as hatched boxes. Nucleotide numbers relative to the left terminus are indicated below the line. Components of the enhancer region (elements I and II) are represented by solid bars above the line.

mutants is also observed with viruses that contain mutations outside of the element II region. Our data indicate that the element II phenotype may reflect a spacing requirement between sequences within and outside of the E1A enhancer region.

MATERIALS AND METHODS

Mutant plasmids and viruses. Ad5 *dl*309 (21) is a phenotypically wild-type virus that is the parent of all of the viruses used in this study. *dl*309 contains a unique *Xba*I cleavage site at 3.8 map units. Plasmid pE1A-WT contains the left-end *Xba*I fragment from *dl*309 (0 to 3.8 map units) cloned into pBR322 (17). All of the mutants analyzed in this study were originally constructed in plasmid pE1A-WT, and the mutations were subsequently rebuilt into intact viruses by the method of Stow (34). Mutant viruses were propagated in cells from cell line 293, a human cell line which expresses the Ad5 E1A products (13).

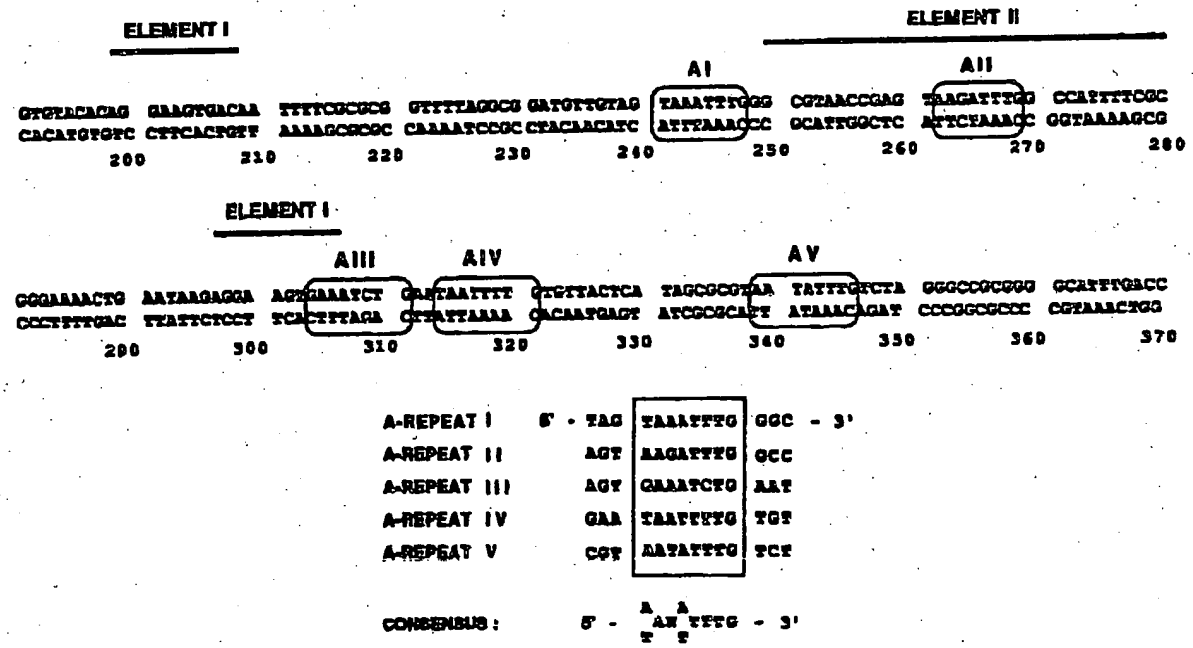

FIG. 2. Nucleotide sequence of the packaging-enhancer region. Repeats AI through AV are indicated. Numbers relative to the left terminus are indicated below the sequence. Enhancer elements I and II are indicated by solid bars above the sequence. A sequence comparison of the individual A repeats is shown below the packaging-enhancer region sequence. The A repeat consensus sequence is given.

A unique *Xho*I linker was inserted in plasmid pE1A-WT at either the *Rsa*I site at nt 194 or the *Sst*II site at nt 358. Unidirectional deletion mutations were introduced into these plasmids, progressing from the unique *Xho*I site in either plasmid and extending into the packaging domain. Deletion mutations were generated, using exonuclease III and S1 nuclease, as described previously (25); *Xho*I linkers were introduced at each deletion endpoint. The endpoint of each mutation was determined by nucleotide sequence analysis, using the dideoxy sequencing procedure (30). Deletion mutations *dl*A5, *dl*18, and *dl*2 in the packaging domain were described previously (16–18).

Plasmids containing double mutations that combine selective single deletions were generated, using the unique *Bal*I site at nt 270 (or the *Hae*III site at nt 270) and unique *Eco*RI site in pBR322 to interchange left-end DNA fragments. Insertions mutations *dl*194/310:*in*4 and *dl*194/316:*in*4 were generated by linearizing the parental mutant plasmid with *Xho*I, followed by a repair reaction with Klenow DNA polymerase and subsequent ligation.

A 31-bp oligonucleotide that corresponds to A repeat II (nt 252 to 275) and that contains cohesive *Xho*I ends was introduced into a derivative of pE1A-WT that lacks the packaging domain (nt 194 to 358; equivalent to mutant *dl*2 previously described [17]) and contains an *Xho*I linker at the deletion junction. The oligonucleotides were inserted in a head-to-tail and a direct orientation with respect to the orientation of A repeat II in the wild-type virus. The sequence of the oligonucleotide is 5′-TCGAGGTAACCGAG TAAGATTTGGCCATTCC-3′:5′-TCGAGGAATGGCCAA ATCTTACTCGGTTACC-3′.

Cells and determination of virus yields and packaging efficiency. Cell line 293 (13) was maintained in Dulbecco modified minimal essential medium containing 10% calf serum. All virus infections were performed at a multiplicity of infection of 3 to 5 PFU per cell at 37°C for 1 h. After infection, the cells were washed twice with phosphate-buffered saline solution, and fresh medium was added. For the determination of virus yields in single-virus infections, infected cultures were harvested 48 h after infection, the cells were lysed by three cycles of freezing and thawing, and infectious virus yields in cleared lysates were determined by plaque assay on 293 cells. With experiments involving mixed-virus infections, 293 cells were infected with 3 to 5 PFU of each virus per cell, as described above. In all of the experiments described, *dl*309 was used as the coinfecting wild-type virus. At 48 h after infection, one-half of the cells were used to isolate high-molecular-weight DNA, and the other half of the cells were used to prepare viral DNA from virions. For the isolation of infected-cell high-molecular-weight DNA, the cells were lysed by the addition of Nonidet P-40 to 0.4%, the nuclei were precipitated, and high-molecular-weight DNA was isolated as described previously (26). For the isolation of viral DNA from virions, the procedure described by Hammarskjold and Winberg (14) was used, with the following modifications. Infected cells were precipitated and suspended in lysis buffer (20 mM Tris [pH 9.0], 0.2% deoxycholate, 10% ethanol). After incubation for 60 min at room temperature, the lysate was cleared at 10,000 × *g* for 30 min. The supernatant was adjusted to 2 mM $CaCl_2$ and 2 mM $MgCl_2$ and was digested with 40 μg of RNase A per ml and 10 μg of DNase I per ml at 37°C for 30 min. The reaction was stopped by the addition of EDTA and EGTA to a final concentration of 5 mM each. Virus particles were lysed by the addition of Sarkosyl to 0.5%, and the samples were digested with 1 mg of pronase per ml at 37°C for 1 h to several hours. After extensive phenol-chloroform extractions, the viral DNA was precipitated with ethanol. DNAs isolated from nuclei or virions were digested with *Cla*I and *Xho*I and analyzed by Southern hybridization (26). The plasmid pE1A-WT, ^{32}P labeled by the random primer method (11), was used as a probe in the Southern hybridization analyses. The relative intensities of the bands in autoradiograms were determined by laser densitometetry, using blots that were exposed to X-ray film without an intensifying screen. The data presented for virus yields from single infections represent the averages of three independent experiments. The data presented for packaging efficiency based on coinfection experiments represent the averages of three to five independent experiments.

The level of the input viral genomes was analyzed by slot blot analysis as previously described (19). 293 cells were infected with 3 to 5 PFU of each virus per cell, and high-molecular-weight DNA was prepared from isolated nuclei at 6 h after infection. The level of total input viral DNA was quantitated by slot blot analysis, using a ^{32}P-labeled probe of the Ad5 genome.

RESULTS

Construction and analysis of viruses with mutations in the packaging domain. To further define the sequences that are required in *cis* for the efficient packaging of Ad5 DNA into virions, we constructed a series of viral mutants that contain single- and double-deletion mutations in the previously defined packaging domain (Ad5, nt 194 to 358) (Fig. 1) (16, 17). Unidirectional deletions that progress into the packaging domain that start at either the upstream border (nt 194) or the downstream border (nt 358) of this region were generated. Combinations of specific mutations of interest were also constructed. Viral mutants were propagated and titrated in 293 cells (13). This cell line expresses the E1A gene products and complements the packaging domain mutants that express reduced E1A levels due to mutations that affect the E1A enhancer sequences. A number of the viral mutants produced small plaques. To verify that each of the mutant stocks was titrated accurately, 293 cells were infected with the viral mutants, and viral DNA present in the nucleus at 6 h after infection was analyzed by slot blot analysis. Each of the mutant viruses displayed comparable levels of nuclear DNA (within twofold of each other) at early times after infection (data not shown).

Two independent assays were used to analyze the efficiency of packaging with the viral recombinants. First, the mutant viruses were used in single infections in 293 cells, and the infectious virus yield obtained after 2 days was determined by a plaque assay. As described above, viruses with mutations in enhancer element II synthesize reduced levels of the early gene products and consequently display a three- to sevenfold reduction in virus yield (termed the element II phenotype) (16, 18). This effect is independent of viral packaging. The reduced yield with enhancer element II mutants is complemented in *trans* in a mixed infection with a wild-type virus, since the coinfecting virus provides normal levels of the viral early gene products (16, 18). To determine what portion of a reduction in a virus yield was the result of a packaging defect, we performed a second assay that used a coinfection with a wild-type virus (the parental virus *dl*309). In these experiments, 293 cells were coinfected with a mutant and wild-type virus. At 2 days after infection, one-half of the infected cells were used to prepare

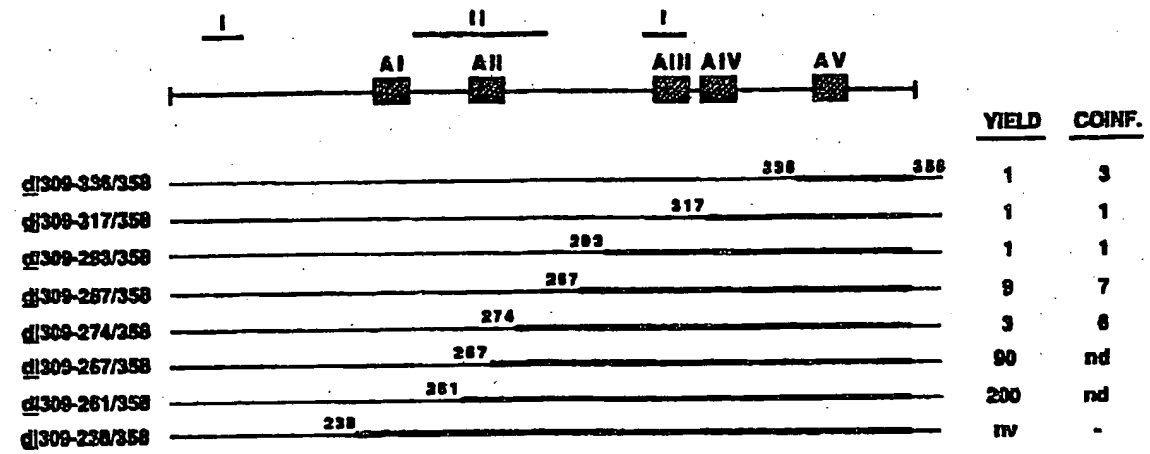

FIG. 3. Schematic view of viral mutants that carry deletions with a common start site at nt 358. The top of the figure positions A repeats I through V and the enhancer elements. The individual deletion mutant names are given on the left. The nucleotide numbers correspond to the first nucleotides present on either side of the deletion. The deleted sequences are indicated by solid bars. For single-virus infections, 293 cells were infected with the individual mutant viruses, and the infectious virus yield was determined by a plaque assay with cellular extracts prepared 48 h after infection. Mutant virus yields (YIELD) are expressed as the fold reduction in yield relative to that of the wild-type virus (yield wild type/yield mutant virus ratio). NV, Nonviable mutant virus. In coinfection experiments, 293 cells were coinfected with a wild-type virus (*dl*309) and the individual mutant viruses. At 48 h after infection, high-molecular-weight DNA was prepared from isolated nuclei and encapsidated viral DNA was prepared from virion particles. Wild-type and mutant viral DNAs in each preparation were distinguished by restriction enzyme digestion and Southern hybridization analysis (Fig. 4). Mutant virus packaging efficiency (COINF.) is expressed as the fold reduction in packaged mutant DNA relative to the packaged coinfecting wild-type DNA. These data were normalized to the amount of each viral DNA (mutant and wild type) present in total nuclear DNA. ND, Encapsidated mutant viral DNA was below the level of accurate quantitation; –, not done.

nuclear high-molecular-weight DNA, and the other half of the cells were used to prepare viral DNA from virion particles. The coinfecting input viral genomes were distinguished by restriction endonuclease digestion and Southern hybridization analysis. By comparing the relative amounts of mutant and wild-type viral DNA present in the nucleus of infected cells with the relative amounts of each viral DNA present in intact virions, we could accurately measure the packaging efficiency of mutant viruses, independent of any effect of enhancer element II.

The packaging domain contains functionally redundant elements. The first set of mutant viruses contains unidirectional deletions which progress from a common site at nt 358 towards the upstream border of the packaging domain (Fig. 3); the data obtained with these mutants in viral infections are shown in Fig. 3 and 4. Viruses containing deletions that extend from nt 358 to 293 grew as well as the wild-type virus did when they were assayed in single infections or in coinfections with a wild-type virus (Fig. 3, *dl*309-336/358, *dl*309-317/358, and *dl*309-293/358). The deletion of sequences between nt 293 and 274 resulted in a three- to ninefold decrease in virus yield in a single infection and an approximate sixfold decrease in a coinfection (Fig. 3, *dl*309-287/358 and *dl*309-274/358). The deletion of an additional 7 nt beyond the endpoint in mutant *dl*309-274/358 resulted in a dramatic decrease in packaging efficiency. Mutant *dl*309-267/358 was reduced nearly 100-fold in virus yield in a single infection (Fig. 3), and no detectable signal was observed in packaged virion DNA in a coinfection (Fig. 4). This mutant replicated to the level of the wild-type virus, however, when nuclear DNA was examined, demonstrating that this effect was due to a packaging deficiency (Fig. 4). The deletion of an additional 6 nt beyond the *dl*309-267/358 endpoint resulted in a similar defective phenotype (Fig. 3, *dl*309-261/358). The deletion of sequences to nt 238 resulted in the loss of virus viability (Fig. 3, *dl*309-238/358). On the basis of control titration experiments, we estimate that the coinfection experiments detected mutants with an approximate reduction of up to 25-fold in packaging efficiency. In repeated reconstruction experiments, we found that mutants that displayed an approximate decrease greater than 1,000-fold in virus yield were nonviable. From the analyses with this group of mutants, we conclude that essential sequences for viral packaging are located between nt 238 and 293 and that this region appears to be composed of three distinct elements located between nt 238 and 261, nt 261 and 274, and nt 274 and 293. Two of these subregions correspond to A repeats I and II (Fig. 1 and 2) that have been implicated in the Ad5

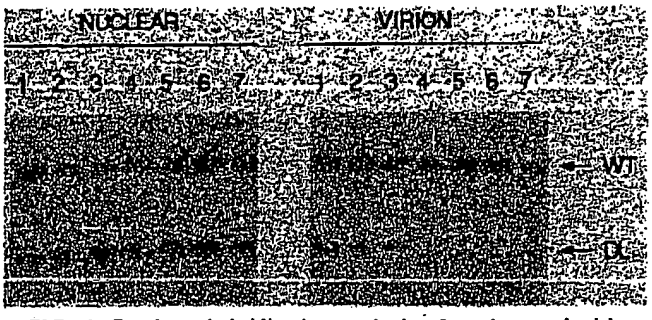

FIG. 4. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfected with the wild-type (*dl*309) and individual mutant viruses (Fig. 3). Nuclear high-molecular-weight DNA and virion DNA were digested with *Cla*I and *Xho*I and analyzed by Southern hybridization, using the Ad5 left-end *Xba*I fragment (plasmid pE1A-WT) as a ^{32}P-labeled probe. The corresponding wild-type (WT) and mutant (DL) left-end DNA fragments are indicated. The mutant viruses tested were *dl*309-336/358 (lane 1), *dl*309-317/358 (lane 2), *dl*309-293/358 (lane 3), *dl*309-287/358 (lane 4), *dl*309-274/358 (lane 5), *dl*309-267/358 (lane 6), and *dl*309-261/358 (lane 7).

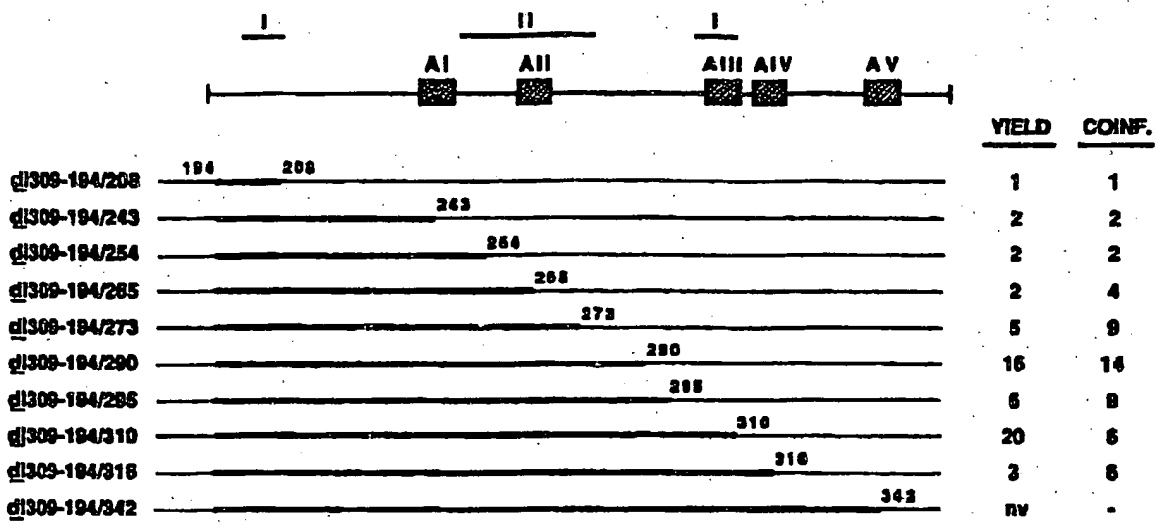

FIG. 5. Schematic view of viral mutants that carry deletions with a common start site at nt 194. The schematic, mutant names, endpoints of the deletions, and in vivo packaging analyses are as described in the legend to Fig. 3.

packaging process (16). In addition, the region between nt 274 and 293 contributes to efficient viral packaging.

The second set of mutant viruses contain unidirectional deletions which progress from a common site at nt 194 towards the downstream border of the packaging domain (Fig. 5); the data obtained with these mutants in viral infections are shown in Fig. 5 and 6. Mutants carrying deletions of sequences between nt 194 and 254, including A repeat I, grew nearly as well as the wild-type virus in a single infection or coinfection (Fig. 5, *dl*309-194/208, *dl*309-194/243, and *dl*309-194/254). The additional deletion of A repeat II resulted in a two- to fivefold decrease in yield in a single infection and a four- to ninefold reduction in packaged DNA in a coinfection (Fig. 5, *dl*309-194/265 and *dl*309-194/273). Thus the region that was critical for viral packaging on the basis of results obtained with the first set of mutant viruses had only a moderate effect on packaging when the downstream half of the packaging domain was intact. The larger decrease observed in a coinfection relative to a single infection may represent a competition between the coinfecting viruses for a limiting factor(s) required for packaging (see Discussion). The deletion of sequences between nt 273 and 316 had no additional effect on packaging when assayed in a coinfection experiment (Fig. 5, mutants *dl*309-194/290 through *dl*309-194/316). However, reproducible fluctuations were observed with these mutants when virus yields in single infections were examined (5-, 16-, 6-, 20-, and 3-fold decreases in yield for mutants *dl*309-194/273, *dl*309-194/290, *dl*309-194/295, *dl*309-194/310, and *dl*309-194/316, respectively) (Fig. 5). This phenotype will be discussed in greater detail below. When nuclear DNA was examined in coinfections, each of these mutants replicated to a level comparable to that of the wild-type virus (Fig. 6). Finally, the deletion of sequences between nt 316 and 342 resulted in the loss of virus viability (Fig. 5, *dl*309-194/342); this region contains A repeats IV and V. We conclude from the analyses of both sets of mutants that the Ad5 packaging domain contains redundant packaging signals.

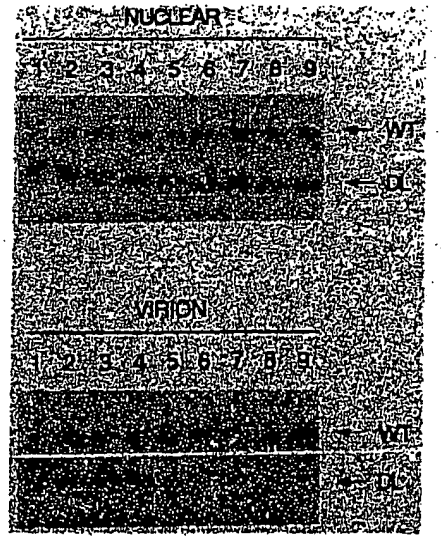

FIG. 6. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfected with the wild-type (*dl*309) and individual mutant viruses (Fig. 5). Southern hybridization analysis of nuclear high-molecular-weight DNA and virion DNA was performed as described in the legend to Fig. 4. The mutant viruses tested were *dl*309-194/208 (lane 1), *dl*309-194/243 (lane 2), *dl*309-194/254 (lane 3), *dl*309-194/265 (lane 4), *dl*309-194/273 (lane 5), *dl*309-194/290 (lane 6), *dl*309-194/295 (lane 7), *dl*309-194/310 (lane 8), and *dl*309-194/316 (lane 9).

On the basis of these results, we created two series of double mutants that combine individual deletions from the sets of mutants described above; a schematic view of these mutants is shown in Fig. 7. The first series is composed of mutants which contain a deletion of the downstream half of the packaging domain (nt 274 to 358) in addition to deletions that remove increasing amounts of the upstream portion of this region. The second series is composed of mutants which contain a deletion of the upstream half of the packaging domain (nt 194 to 271) in addition to deletions that remove increasing amounts of the downstream portion of this region. The packaging efficiency of these mutant viruses was examined in single infections and coinfections with a wild-type virus. The data obtained from viral infections with these mutants are shown in Fig. 7.

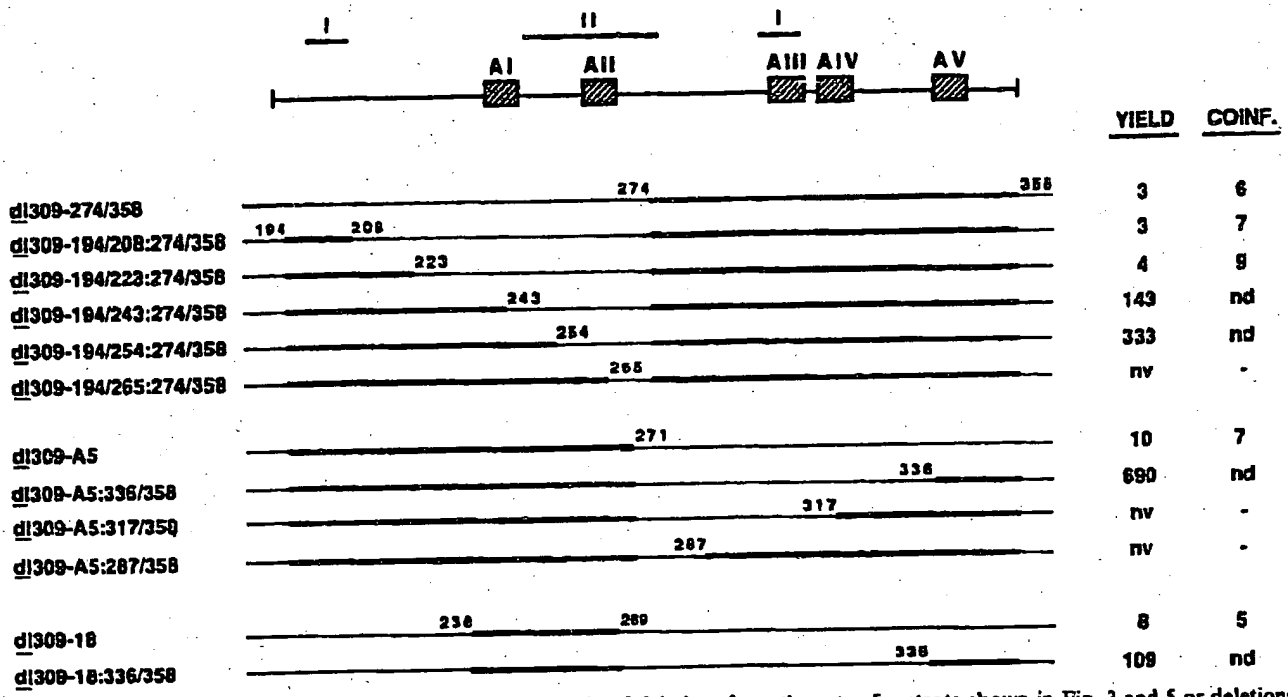

FIG. 7. Schematic view of viral mutants that combine individual deletions from the sets of mutants shown in Fig. 3 and 5 or deletions mutants *dl*309-A5, *dl*309-18, and *dl*309-9 that were previously described (16–18). The schematic, mutant names, endpoints of the deletions, and in vivo packaging analyses are as described in the legend to Fig. 3.

With double mutants in the first series (*dl*309-274/358 background), the deletion of sequences between nt 194 and 223 did not reduce packaging efficiency further than the parental mutant virus did (Fig. 7, *dl*309-274/358 versus *dl*309-194/208:274/358 and *dl*309-194/223:274/358). However, the deletion of sequences to nt 243, including part of A repeat I, resulted in an approximate 40-fold decrease in virus yield in a single infection (Fig. 7, *dl*309-194/243:274/358). The complete deletion of A repeat I resulted in a similarly defective virus (Fig. 7, *dl*309-194/254:274/358). Neither of the latter two mutants displayed detectable packaged viral DNA in a coinfection assay; both mutants, however, exhibited wild-type levels of nuclear DNA (data not shown). The additional deletion of sequences that include A repeat II resulted in the loss of virus viability (Fig. 7, *dl*309-194/265:274/358). With double mutants in the second series (*dl*309-A5 background), the deletion of sequences between nt 336 and 358, including A repeat V, resulted in a 70-fold decrease in virus yield in a single infection compared with the yield from the parental mutant virus (Fig. 7, *dl*309-A5 versus *dl*309-A5:336/358). No viral DNA was detectable in virions in a coinfection experiment using this double mutant, although this virus replicated to a level comparable to that of the wild-type virus (not shown). The additional deletion of sequences to nt 317, including A repeat IV, resulted in the loss of virus viability (Fig. 7, *dl*309-A5:317/358).

The phenotype of mutant viruses in the second series of double mutants was attributable, at least in large part, to A repeats I and II in the upstream half of the packaging domain. This conclusion is based on the analysis of an additional double mutant that combines a smaller deletion of A repeats I and II (Fig. 7, *dl*18) with the deletion of A repeat V (*dl*309-18:336/358). Mutant *dl*309-18 was reduced fivefold for packaging (Fig. 7). The additional deletion of A repeat V in conjunction with the deletion of A repeats I and II resulted in a 13-fold decrease in virus yield in a single infection and the lack of detectable packaged DNA in a coinfection experiment (Fig. 7) (data not shown). Each of these mutant viruses replicated to a level comparable to that of the wild-type virus in a coinfection (data not shown).

The results obtained with the double mutants strongly support the idea that the A repeats constitute redundant functional elements of the Ad5 packaging domain. In the absence of A repeats III, IV, and V, A repeats I and II provided sufficient, albeit reduced, packaging functions. This residual packaging efficiency was greatly reduced with the deletion of A repeat I, and viruses that lacked all A repeats were nonviable. Similarly, in the absence of A repeats I and II, residual packaging activity was provided by A repeats IV and V. The deletion of A repeat V resulted in a dramatic decrease in packaging efficiency, and a virus that lacked A repeats I, II, IV, and V was nonviable.

Late viral protein synthesis was examined with a representative set of the mutants described above. Each of the mutant viruses accumulated viral late polypeptides at levels that were within twofold of that of the wild-type virus (data not shown). Since each of these viruses replicated viral DNA at normal levels in coinfection experiments and synthesized normal levels of viral late proteins in single infections, we conclude that the defect observed with these mutants occurs late in the lytic cycle. This conclusion is consistent with a defect in the packaging of viral DNA into virions.

Insertion of an A repeat oligonucleotide in place of the packaging domain. Our model suggests that the A repeats function to enhance viral packaging and have an additive effect on packaging efficiency. To test this model further, we constructed recombinant viruses that contain one or more copies of an oligonucleotide to A repeat II (nt 252 to 275) in place of the entire packaging domain. The data obtained from infections with these recombinant viruses are shown in

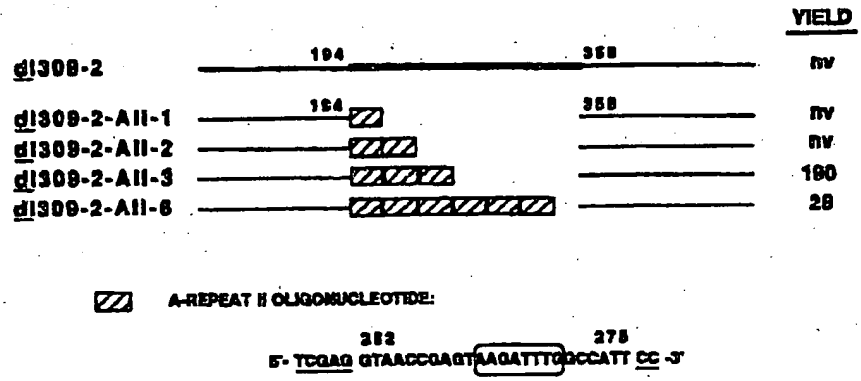

FIG. 8. Analysis of viral mutants that contain one or more copies of an oligonucleotide to A repeat II (the sequence is shown below the mutant diagram) in place of the entire packaging domain (mutant *dl*309-2 background). The solid bar represents the deletion in mutant *dl*309-2. The hatched boxes represent the A repeat II oligonucleotide(s) present in mutants *dl*309-2-AII-1 (1 oligonucleotide), *dl*309-2-AII-2 (2 oligonucleotides), *dl*309-2-AII-3 (3 oligonucleotides), and *dl*309-2-AII-6 (6 oligonucleotides). Infectious virus yields (YIELD) in single infections of 293 cells were determined as described in the legend to Fig. 3.

Fig. 8. The insertion of one or two A repeat oligonucleotides in place of the packaging domain did not restore virus viability. A virus that contained three repeated oligonucleotides was viable but was reduced approximately 200-fold in virus yield in a single infection compared with the wild-type virus. A virus that contained six copies of the A repeat oligonucleotide was also viable and was reduced 28-fold in virus yield compared with the wild-type virus. We could not detect packaged DNA in a coinfection experiment with either viable virus (data not shown). These results demonstrate that a single A repeat (A repeat II), when multimerized, can rescue the packaging defect, although these recombinant viruses were clearly still defective compared with the wild-type virus.

A spacing phenomenon correlates with an enhancer element II phenotype. A number of the mutants had an apparent spacing effect on virus yield in a single infection. This was particularly evident with viruses that contain unidirectional deletions that progress from nt 194 towards the downstream border of the packaging domain. Specifically, a cyclic effect on virus yield was observed with mutants *dl*309-194/273 through *dl*309-194/316 (Fig. 5). We were intrigued by the fact that all of the mutants that exhibited less severe growth defects contain deletions in which the variable 3' endpoints were separated from each other by nearly integral turns of the helix (*dl*309-194/273, *dl*309-194/295, and *dl*309-194/316). The 3' endpoints of deletions with mutants that displayed greater reductions in virus yields were placed on the opposite face of the DNA helix (*dl*309-194/290 and *dl*309-194/310). This result suggested that the fluctuations in virus yield observed with these mutants (similar to the enhancer element II phenotype described previously [18]) reflect a specific spacing requirement between one or more elements that are located within or outside of the enhancer region. This possibility was supported by the data obtained in the coinfection experiments in which each of these viruses was reduced in packaging efficiency to a similar degree (Fig. 5). The element II phenotype would be complemented by the coinfecting wild-type virus in these experiments (16, 18).

To test this model, we constructed two additional mutant viruses that alter the spacing in the packaging-enhancer region. By repairing the *Xho*I linker present at the junction of the deletion endpoints, we created two mutants, *dl*309-194/310:*in*4 and *dl*309-194/316:*in*4, that differ from their respective parental viruses by a 4-bp insertion (Fig. 9). We reasoned that if the higher yield obtained with mutant *dl*309-194/316 reflects a favorable spacing arrangement in the enhancer region, the insertion of 4 bp would reduce the virus yield obtained with this mutant. Similarly, if the reduced yield obtained with mutant *dl*309-194/310 reflected an unfavorable spacing arrangement in the enhancer region, the insertion of 4 bp may increase the virus yield obtained with this mutant. The data obtained with the insertion mutants in single infections and coinfections are shown in Fig. 9. In single infections, the yields obtained with the parental viruses *dl*309-194/310 and *dl*309-194/316 were reduced 20- and 3-fold, respectively, compared with yields obtained with the wild-type virus. With mutant *dl*309-194/310:*in*4, the yield obtained in a single infection was reduced sixfold compared with the yield from the wild-type virus, a threefold increase relative to the parental mutant. Similarly, with mutant *dl*309-194/316:*in*4, the yield obtained in a single infection was

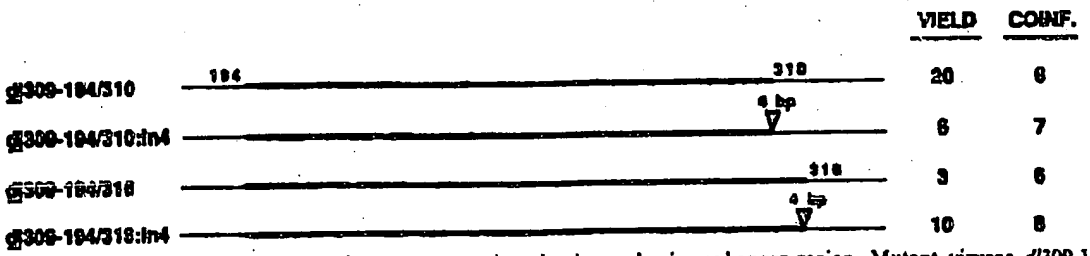

FIG. 9. Analysis of viral mutants that contain spacer mutations in the packaging-enhancer region. Mutant viruses *dl*309-194/310 and *dl*309-194/316 are the same as those shown in Fig. 5. The 4-bp spacer mutations were inserted at the deletion endpoints (triangles). The in vivo packaging analyses (YIELD and COINF.) were performed as described in the legend to Fig. 4, except that the isolated DNAs were digested only with *Cla*I for Southern hybridization analysis.

reduced 10-fold compared with the yield from the wild-type virus, a threefold decrease relative to the parental mutant virus. In contrast, each of these mutant viruses displayed a comparable decrease in packaging efficiency when assayed in a coinfection experiment. These results support the notion that the enhancer element II phenotype observed with various mutant viruses in these studies and in previous analyses (18) reflects, at least in part, a spacing constraint of sequences at the left end of the Ad5 genome that influence enhancer region activity. The nature of these sequences is unknown.

DISCUSSION

On the basis of the results obtained with wild-type adenovirus and several temperature-sensitive mutants, it is generally assumed that adenovirus packages its DNA into preformed empty capsids, the proheads (4, 6, 7, 10, 20, 36). The demonstration of polarity in the packaging process (5, 14, 15, 37) and the delimitation of packaging domains in several adenovirus genomes (14, 16, 29) suggest that the process by which viral DNAs are selected from a pool of intracellular DNAs for packaging involves the recognition of specific viral sequences by a packaging protein(s). This was clearly shown for the packaging of bacteriophage lambda DNA in which a phage-encoded protein, terminase, recognizes and binds to a specific viral DNA sequence termed the *cos* site (12, 31). The terminase-lambda DNA complex then binds to the proheads, and packaging ensues (12). In other systems (for example, phages T7, T3, or p22), similar specific DNA-protein interactions are required to ensure proper packaging of the phage chromosome (8). *cis*-Acting packaging regions have also been shown to be present in the genomes of several retroviruses (1, 22, 24, 27, 32, 39) and herpesviruses (33, 35, 38). In these cases, specific proteins that interact with these sites have not been identified.

Here we report the identification of *cis*-acting elements that are required for efficient encapsidation of the Ad5 genome. Our results show that the packaging signal is composed of at least five elements which are located within the packaging domain between nt 238 and 261, nt 261 and 274, nt 274 and 293, nt 317 and 336, and nt 336 and 358. Four of five of these regions contain one copy of a repeated sequence, termed the A repeat (16), with the consensus sequence 5'-$\overset{A}{T}$ANA$\overset{A}{T}$TTG-3' (Fig. 2). The region between nt 274 and 293 does not contain any obvious sequence similarity with the repeated element, aside from the fact that it is also very AT rich (Fig. 2). Other AT-rich sequences are located in the packaging domain (nt 208 to 215 and nt 361 to 368); a role for these sequences in packaging has not been determined. Our results strongly suggest that the A repeat represents a bona fide packaging element. Viruses that contained either the region with A repeats I and II (nt 238 to 274) or A repeats IV and V (nt 316 to 358) were reduced approximately sixfold in viral packaging in coinfection experiments (Fig. 3, *dl*309-274/358; Fig. 5, *dl*309-194/316; Fig. 7, *dl*309-194/223:274/358 and *dl*309-A5). The deletion of an additional A repeat with any of these mutants greatly reduced packaging efficiency (Fig. 3, *dl*309-267/358 and *dl*309-261/358; Fig. 7, *dl*309-194/243:274/358, *dl*309-194/254:274/358, and *dl*309-A5:336/358). The functional redundancy of individual elements in the packaging process is consistent with the role of a repeated sequence in this process. On the basis of these results, the A repeats appear to be redundant and have an additive effect on packaging efficiency. The additive effect of individual A repeats was also evident with recombinant viruses that contained multiple copies of A repeat II (Fig. 8). The fact that the recombinant virus that carries six copies of A repeat II did not package viral DNA more efficiently may reflect the need for a particular spacing of adjacent A repeat motifs with respect to each other or the possibility that a functional hierarchy exists within the A repeat family. With Ad3, the region between nt 319 and 390 specifies polar packaging (29). The comparable region in Ad5 contains A repeats IV and V (Fig. 2), which are conserved between the Ad3 and Ad5 genomes (23). This suggests that the packaging of other adenovirus serotypes also relies on elements that are similar to the Ad5 A repeat sequence.

On the basis of the results obtained in these studies, we suggest the following model to account for the selectivity of the packaging process. In analogy to the phage lambda system, the individual packaging elements may represent the specific binding sites for a packaging protein(s). The binding of this protein(s) to these sites would allow the viral genome to recognize and position itself properly with a prohead, and packaging may ensue. It is possible that the redundancy of the packaging elements simply increases the number of binding sites in the viral genome to increase the probability of factor binding to viral DNA, as opposed to cellular sequences. This phenomenon has been clearly demonstrated with the repeated 60/81-bp elements in the *Xenopus* ribosomal RNA promoter region and has been termed the sink effect (28). This possibility is consistent with the results obtained with a number of mutants in which a greater decrease in packaging efficiency in coinfection experiments was evident in comparison with the results obtained with the same mutant viruses in single infections. We speculate that the greater reduction observed in the coinfection with these mutants represents a competition between the wild-type and mutant genomes for limiting concentrations of a packaging protein(s). Since the mutant genome contains fewer binding sites, it is a weaker competitor for factor binding. This competitive effect is also reminiscent of results obtained with the *Xenopus* repetitive ribosomal gene array (28).

It is also possible that the repeated packaging elements represent an array of interspersed binding sites whereby the binding of appropriate proteins would result in the formation of a defined DNA-protein structure. Only molecules bearing this structure would be suitable substrates for the packaging process. Both of these models imply that the packaging elements represent the binding sites for a specific protein(s) involved in the packaging process and that the specific DNA-protein interaction provides the molecular basis for the observed packaging efficiency. However, taking into account that the fifth packaging element (nt 274 to 293) does not contain any primary sequence homology to the A repeats aside from its AT-rich character, another model to account for the packaging process can be established. It is known that AT-rich domains alter the overall conformation of a DNA molecule by introducing bends in the helix (40). Restriction fragments that span the packaging-enhancer region show abnormal migration behavior in polyacrylamide gels, a result which is consistent with bent DNA (Gräble and Hearing, unpublished results). The presence of bending sites in the left end of the Ad5 genome was also recently reported (2, 9). Three of the bending loci proposed in this study coincide with A repeats I, IV, and V (2, 9). Therefore, it is conceivable that a packaging protein(s) recognizes the overall structure of the DNA molecule, represented as bent DNA, or that this structure is required for recognition of a prohead.

A number of mutant viruses displayed a complex pheno-

type which showed an apparent contradiction between the results obtained in single infections and those obtained in coinfection experiments (Fig. 5, mutants *dl*309-194/273 through *dl*309-194/316). Several of these mutants exhibited packaging defects in coinfections which could account for only a portion of the defect observed in virus yield in a single infection. These results indicated that the introduced deletion, aside from reducing the packaging capacity of the mutant genome, also impaired an additional function required for normal virus replication. Since the presence of a wild-type virus in the coinfection experiment can compensate for this additional defect, the defect is probably the result of the lack of a *trans*-acting factor. At the present time, the nature of this defect is unknown. However, since the phenotype of these mutants is reminiscent of the phenotype displayed by enhancer element II mutants previousiy described (18), we believe that the additional growth defect with these mutants might be caused by the inactivation of an enhancer element. Enhancer element II was previously mapped to Ad5 nt 250 to 280 (18). Our results, however, indicate that the regulatory element(s) responsible for this effect may be located outside of the E1A enhancer region, since the fluctuation in virus yield in single infections was clearly related to a spacing effect of flanking regions (Fig. 9, *dl*309-194/310 versus *dl*309-194/310:*in*4 and *dl*309-194/316 versus *dl*309-194/316:*in*4). This does not exclude a role for the region located between nt 250 and 280, but additional flanking elements are probably involved in this phenomenon. The nature of these elements is unknown.

The organization of the Ad5 packaging-enhancer region is complex, with redundant as well as overlapping transcriptional and packaging regulatory elements. The binding of specific proteins to the packaging sequences at late times after infection may displace or prevent the interaction of transcriptional components with this region. The overlapping organization of enhancer and packaging sequences may have evolved to repress transcription at late times after infection when packaging occurs. This may alter the conformation of viral DNA from an open conformation involved in transcription to a closed conformation to elicit viral DNA condensation by the core and auxiliary proteins. Thus, the packaging process may represent an additional level at which viral expression is temporally regulated.

ACKNOWLEDGMENTS

We thank our colleagues for many helpful discussions and Tina Philipsberg for excellent technical help.

This research was supported by Public Health Service grant CA28146 from the National Cancer Institute.

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JOURNAL OF VIROLOGY, Feb. 1992, p. 723–731
0022-538X/92/020723-09$02.00/0

Vol. 66, No. 2

cis and *trans* Requirements for the Selective Packaging of Adenovirus Type 5 DNA

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Received 29 August 1991/Accepted 28 October 1991

Polar packaging of adenovirus DNA into virions is dependent on the presence of *cis*-acting sequences at the left end of the viral genome. Our previous analyses demonstrated that the adenovirus type 5 (Ad5) packaging domain (nucleotides 194 to 358) is composed of at least five elements that are functionally redundant. A repeated sequence, termed the A repeat, was associated with packaging function. Here we report a more detailed analysis of the requirements for the selective packaging of Ad5 DNA. By introducing site-directed point mutations into specific A repeat sequences, we demonstrate that the A repeats represent *cis*-acting functional components of the packaging signal. Additional elements, located outside the originally defined packaging domain boundaries and that resemble the A repeat consensus sequence, also are capable of promoting the packaging of viral DNA. The *cis*-acting components of the packaging signal appear to be subject to certain spatial constraints for function, possibly reflecting a necessity for the coordinate binding of packaging proteins to these sites. In agreement with this idea, we present evidence that the interaction of a limiting *trans*-acting factor(s) with the packaging domain in vivo is required for efficient encapsidation of the Ad5 genome.

Very little is known about the mechanism that allows selective packaging of the adenovirus (Ad) genome into viral capsids late in the infectious cycle. This event presumably involves the specific recognition of *cis*-acting viral DNA sequences followed by condensation and encapsidation of the viral genome (2, 6–8, 12, 13, 33). It has been shown for adenoviruses in subgroups B (Ad3, Ad7, and Ad16 [3, 19, 25, 30, 34]) and C (Ad5 [3, 20]) that the viral DNA is inserted into preformed empty capsids in a polar fashion from left to right, suggesting the existence of *cis*-acting packaging elements in the left end of the genome. Incomplete particles formed by Ad3 and Ad7 contain subgenomic DNA molecules that are enriched in left-end sequences even though both ends of the viral genome are represented in equivalent quantities in the nuclear pool of subgenomic DNAs (3, 4, 25, 30, 34). Analysis of Ad16 variant viruses that contain different amounts of left-end sequences duplicated at the right end of the genome identified left-end sequences important for selective packaging located between nucleotides (nt) 290 and 390 (19).

We have previously identified a *cis*-acting packaging domain that is located between nt 194 and 358 at the left end of the Ad5 genome (Fig. 1A) (17, 21, 22). Mutants lacking this region are nonviable but can be rescued by insertion of the left-terminal 355 nt at the right end of the genome (22). The Ad5 packaging domain shares properties with eukaryotic transcriptional enhancer elements since the packaging signal functions at either end of the viral genome, in an inverted orientation, and can be moved within several hundred base pairs from its original location without a reduction in virus yield (21). Because of a *cis* requirement for virus replication, it has not been possible to determine whether sequences within the inverted terminal repeats (ITR) are also required for packaging.

The Ad5 packaging domain consists of at least five elements that are functionally redundant (17). Four of the five regions contain an AT-rich repeated sequence motif termed the A repeat (Fig. 1). The fifth region does not contain any obvious primary sequence similarity to the A repeat aside the fact that it is also AT rich. Our results indicated that the A repeats function to enhance viral packaging and that the efficiency of the packaging process is determined by the number of individual elements that are present in the viral genome. On the basis of these results, several models accounting for the selectivity of the encapsidation process can be proposed. It is possible that the individual repeats represent the binding sites for a packaging protein and, considering the repetitive structure of the packaging signal, that the repeated elements form an array of interspersed binding sites whereby the binding of packaging proteins to all the sites would result in the formation of a defined protein-DNA structure. However, given the AT-rich character of the A repeats, it is also conceivable that the function of these elements is to alter the structural conformation of the DNA helix through the introduction of bends (35). Depending on the spatial arrangement of the bending sites, the change in conformation either could be restricted to a confined region such as the AT-rich sequences themselves or could affect the conformation of the left-end portion of the DNA molecule as a whole. In this case, the *cis*-acting recognition signal would be represented by a structural rather than a sequence-specific feature of the viral genome.

The Ad5 packaging domain is located within the early region 1A (E1A) enhancer region (Fig. 1A) (17, 21–23). The E1A enhancer is composed of two functionally distinct enhancer elements that regulate viral gene expression. Enhancer element I is repeated and specifically regulates transcription of the E1A gene (22, 23). Element I mutants are efficiently complemented when the E1A gene products are provided in *trans*. Enhancer element II is located between these repeated sequences and regulates transcription in *cis* of all early regions on the viral chromosome (23). The reduction in viral early gene expression observed with

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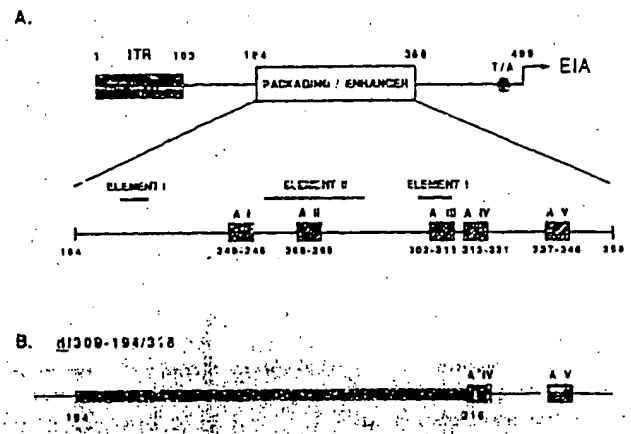

FIG. 1. (A) Schematic diagram of the left end of the Ad5 genome including the E1A 5′-flanking region. Numbers at the top indicate nucleotide positions relative to the left terminus of the genome. The ITR and the packaging/enhancer domain are represented by stippled and open boxes, respectively. The E1A TATA box motif (T/A) is indicated by a black circle. E1A transcription is initiated at nt 499, as indicated by the arrow. An enlarged view of the packaging/enhancer region (nt 194 to 358) is shown below. A repeats AI through AV are represented by the hatched boxes. Their nucleotide positions relative to the left terminus of the genome are indicated by the numbers below the line. Components of the enhancer region (elements I and II [23]) are represented by solid bars above the line. (B) Schematic view of mutant virus *dl*309-194/316. This virus was used as the parental virus for construction of the LS and insertion mutants. The nucleotide numbers correspond to the first nucleotides present on either side of the deletion. The deleted sequences are indicated by the stippled box.

element II mutants results in decreased DNA replication and a corresponding three- to sevenfold reduction in virus yield compared with that of a wild-type virus (21, 23). The *cis*-acting defect observed with element II mutants can be complemented in mixed infections with a wild-type virus in which viral proteins required for efficient propagation are provided in *trans* (17, 21, 23). Our recent data suggest that the originally defined enhancer element II domain (nt 250 to 280) is not sufficient to provide full enhancer activity but that additional flanking sequences are required to ensure proper enhancer function (17). The nature of these elements is still unknown.

Here we describe a more detailed analysis of the *cis* and *trans* requirements for the selective packaging of Ad5 DNA into virions. By introducing site-directed mutations in specific A repeat sequences, we clearly demonstrate that the A repeats represent functional components of the Ad5 packaging signal. Our analysis also identifies additional packaging elements located outside the originally defined packaging domain which resemble the A repeat consensus sequence. Our results suggest that a particular spacing of adjacent packaging elements with respect to each other is required to ensure packaging activity. It is likely that this observed spacing constraint reflects the necessity for the coordinate binding of packaging proteins to these sites. Finally, we present evidence that a limiting *trans*-acting factor(s) interacts with the packaging domain in vivo.

MATERIALS AND METHODS

Mutant viruses and plasmids. Ad5 *dl*309 is a phenotypically wild-type virus that contains a unique *Xba*I cleavage site at 3.8 map units (24). *dl*309-194/316 (Fig. 1B [17]) is a *dl*309 variant that carries a deletion of sequences between nt 194 and 316. Ad5 *dl*10/28 was previously described as *dl*309-194/243:274/358 (17). This double mutant contains a deletion between nt 194 to 242 and between nt 274 to 358 in a *dl*309 background. The linker scanning (LS) mutants (*dl*309-LS) and the spacer mutant *dl*309-380:in6 were originally constructed in plasmid pKS-194/316. This plasmid contains the left-end *Xba*I fragment from *dl*309-194/316 (0 to 3.8 map units) cloned into the *Eco*RI and *Xba*I restriction sites in the polylinker region of the pBS-KS (+) Bluescript vector (pKS; Stratagene). The spacer mutants derived from *dl*10/28 were constructed in plasmid pE1A-10/28 (17), which contains the left-end *Xba*I fragment from *dl*309-194/243:274/358 cloned into pBR322. All mutations were subsequently rebuilt into intact viruses by the method of Stow (32). Mutant viruses were propagated and titered on 293 cells, a human embryonic kidney cell line that constitutively expresses the Ad5 E1A and E1B gene products (18).

A series of LS mutations and an insertion mutation were made by using synthetic oligonucleotides approximately 30 bp in length and complementary to a minimum of 10 bases on each side of the substitution. The names of the LS and insertion mutations and the sequences of the oligonucleotides used to construct them are shown in Table 1. The mutant plasmids were constructed by the method of Kunkel (27). Insertion mutants *dl*10/28-194:in4 and *dl*10/28-358:in4 were generated by partial linearization of the parental plasmid pE1A-10/28 with *Xho*I, followed by a repair reaction with Klenow DNA polymerase and subsequent ligation. The authenticity of each mutation was verified by nucleotide sequence analysis using the dideoxy procedure (31).

Determination of virus yield and packaging efficiency. The 293 cell line (18) was propagated in monolayer cultures in Dulbecco modified Eagle medium supplemented with 10% calf serum. Viral infections of 293 cells were performed at a multiplicity of infection of 5 PFU per cell at 37°C for 1 h. After infection, cells were washed two times with phosphate-buffered saline solution and fresh medium was added.

TABLE 1. Nucleotide sequences of site-directed LS and insertion mutants[a]

Oligonucleotide	Sequence
LS1	5′-GATTTTGTGTTACGTCGACCGCGTAATATTTG-3′ (328–333)
LS4	5′-GTCCTCGAGCGTCGACTGTTACTCATAGC-3′ (316–321)
LS5	5′-CATAGCGCGTAGTCGACGTCTAGGGCC-3′ (340–345)
LS6	5′-GGGGCGCGGGAGTCGACGCGTTTAGGTGG-3′ (363–368)
LS7	5′-CGGGGACTTTGACGTCGACCGTGCGAGACTCGCC-3′ (370–375)
380:in6	5′-CCGTTTACGTGGGTCGACAGACTCGCCC-3′ (380)

[a] The nucleotide sequence flanking each site-directed mutation is shown; each sequence listed represents the oligonucleotide used for mutagenesis. The 6-bp substitutions with the LS mutants and the insertion mutation described in the text are underlined. Numbers in parentheses indicate the location (nucleotides) of the substitution in the Ad5 genome.

For determination of virus yield in a single infection, the infected cell cultures were harvested 48 h postinfection and lysed by three cycles of freezing and thawing. The amount of infectious virus present in these lysates was determined by plaque assays on 293 cells. To determine the packaging efficiency of the mutant viruses, 293 cells were coinfected with 5 PFU of both a mutant virus and wild-type virus *dl*309. At 48 h after infection, one half of the cells was used to isolate total nuclear DNA and the other half was used to prepare viral DNA from virions. For the isolation of infected cell total nuclear DNA, the cells were lysed by the addition of Nonidet P-40 to 0.4%, the nuclei were precipitated, and total nuclear DNA was isolated as described previously (17, 21). For the isolation of viral DNA from virions, the procedure described by Hammarskjold and Winberg (19) was used, with the following modifications (17). Infected cells were precipitated and resuspended in lysis buffer (20 mM Tris [pH 9.0], 0.2% deoxycholate, 10% ethanol). After incubation for 60 min at room temperature, the lysate was cleared at 10,000 × *g* for 30 min. The supernatant was adjusted to 2 mM $CaCl_2$ and 2 mM $MgCl_2$ and was digested with 40 μg of RNase A per ml and 10 μg of DNase I per ml at 37°C for 30 min. The reaction was stopped by the addition of EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) to a final concentration of 5 mM each. Virus particles were lysed by the addition of Sarcosyl to 0.5%, and the samples were digested with 1 mg of pronase per ml at 37°C for 1 h to several hours. After extensive phenol-chloroform extractions, the viral DNA was precipitated with ethanol. DNA isolated from nuclei or virions was digested with *Cla*I and analyzed by Southern hybridization (28) using plasmid pE1A-WT, ^{32}P-labeled by the random primer method (14), as a probe. The relative intensities of the bands in autoradiograms were determined by laser densitometry, using blots that were exposed to X-ray film without an intensifying screen. The data presented for virus yield in the single infections and the data for packaging efficiency based on coinfection experiments represent the averages of four to five independent experiments.

Cotransfection experiment. Monolayer cultures of COS1 cells (16) were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Plasmid pUC-WT-ENH contains the wild-type packaging/enhancer region from Ad5 nt 189 to 355 cloned into the *Sma*I site in the polylinker region of pUC9 (1). Plasmid pOR4 (9) contains simian virus 40 (SV40) sequences 5171 to 160 between the SV40 *Hin*dIII and *Bst*NI sites, which encompass the SV40 origin of replication, and an *Eco*RI linker at the *Bst*NI site. The fragment spanning the origin of DNA replication was excised from vector sequences by *Eco*RI digestion followed by a repair reaction using Klenow DNA polymerase and a second digestion with *Hin*dIII. This fragment was then inserted between the Klenow-repaired *Pst*I site and *Hin*dIII site of the vector plasmid pUC-WT-ENH. The resulting plasmid is referred to as pOR4-PAC$^+$. As a control, we inserted the pOR4-derived *Eco*RI-*Hin*dIII fragment into the same location of a pUC19 plasmid that does not contain the packaging/enhancer region. This control plasmid is referred to as pOR4-PAC$^-$. The authenticity of each plasmid construct was verified by nucleotide sequence analysis (31). DNA transfections of COS1 cells were performed by the calcium phosphate precipitation method (36). At approximately 65% confluency, the cells were transfected with 5 μg of *dl*309 DNA and 15 μg of either pOR4-PAC$^+$, pOR4-PAC$^-$, or salmon sperm carrier DNA per 100-mm diameter dish. After incubation for 12 h with the calcium phosphate precipitate, the cells were washed with Tris-buffered saline solution (TBS), TBS containing 3 mM EGTA, and TBS. Fresh medium was added, and cultures were harvested 36 h later. The cells were lysed by three cycles of freezing and thawing, and infectious virus yields were determined by plaque assay on 293 cells. An aliquot of each culture was used to determine total levels of viral DNA and cytoplasmic late mRNA. Total nuclear DNA was isolated from purified nuclei as described above. The level of total viral DNA was quantitated by slot blot analysis, using a ^{32}P-labeled probe of the Ad5 genome. Total RNA was extracted from cell lysates with guanidinium thiocyanate followed by centrifugation over cesium chloride (28). The level of viral late mRNAs were quantitated by slot blot analysis using a ^{32}P-labeled probe corresponding to Ad5 late region L3 (52.5 to 58.5 map units).

RESULTS

Construction and analysis of viruses containing LS mutations in the packaging domain. To further test the involvement of the A repeats in the encapsidation process, we constructed a series of viral mutants in which individual A repeats were specifically inactivated by the introduction of a 6-bp LS mutation. In view of the functional redundancy of the packaging elements, the mutations were introduced into a mutant virus background (*dl*309-194/316; Fig. 1B) (17) containing a deletion of A repeats I, II, and III. Recombinant viruses (Fig. 2) were constructed that contain LS mutations in A repeat IV (*dl*309-LS4, nt 316 to 321), in A repeat V (*dl*309-LS5, nt 340 to 345), and in the spacer region between A repeats IV and V (*dl*309-LS1, nt 328 to 333). Two independent assays were used to test the efficiency of packaging with the mutant viruses. First, overall virus growth was determined in single infections of 293 cells. As described above, viruses containing mutations that affect enhancer element II function show reduced expression of the early gene products which results in decreased viral DNA replication and a corresponding reduction in virus yield (23). This effect, referred to as the element II phenotype, is independent of viral packaging and can be efficiently complemented when viral gene products required for replication are provided in *trans* by a coinfecting wild-type virus (17, 21). To determine what portion of the observed reduction in virus yield was due to a packaging defect, mutant viruses were analyzed in a coinfection experiment with the wild-type parental virus *dl*309. In these experiments, 293 cells were coinfected with a mutant and a wild-type virus. Two days after infection, one half of the cells was used to isolate total nuclear DNA from purified nuclei and the other half was used to isolate encapsidated viral DNA from purified virus particles. Both coinfecting genomes were distinguished by restriction enzyme digestion followed by Southern hybridization analysis. By comparing the relative amounts of mutant and wild-type DNAs in the nuclei of infected cells with the amount of each viral DNA that was actually present in intact virus particles, the packaging efficiency of the mutant genome could be accurately measured independent of an enhancer element II effect.

The parental mutant virus *dl*309-194/316 showed a threefold decrease in virus yield in a single infection and a sixfold reduction in the amount of packaged DNA compared with the wild-type virus in a coinfection (17) (Fig. 2). The data obtained with the LS mutants in A repeats IV and V in single infections and coinfections are shown in Fig. 2 and 3. Mutant viruses *dl*309-LS4 and *dl*309-LS1 grew as well as the parental mutant virus when assayed in single infections or in coinfec-

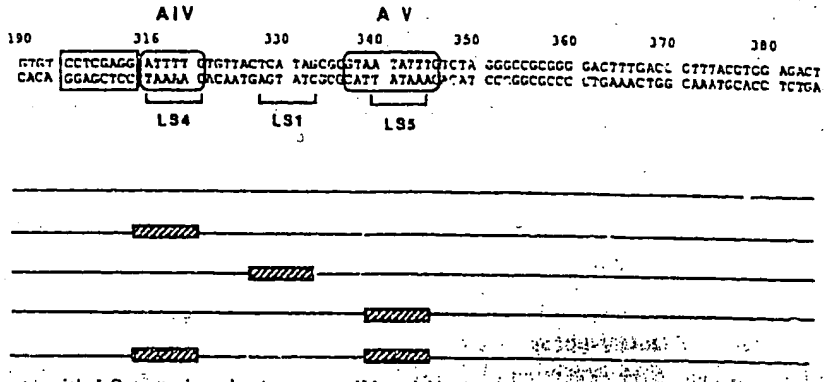

FIG. 2. (A) Viral mutants with LS mutations in A repeats IV and V. At the top is shown the nucleotide sequence of the parent virus *dl*309-194/316 between nt 190 and 385. An *Xho*I linker is present at the deletion junction and is indicated by an open box. Nucleotide numbers relative to the left end are indicated above the line. A repeats IV and V are encircled. The positions and names of the LS mutants are indicated by brackets below the sequence (each LS mutation replaced the wild-type sequence with the sequence GTCGAC). The corresponding single- and double-mutant viruses are listed below, and the LS mutations are indicated by hatched boxes. For single-virus infections, mutant virus yields (YIELD) are expressed as the fold reduction in yield relative to that of the wild-type virus (yield wild type/yield mutant ratio). The total virus yield in an infection with the wild-type virus (*dl*309) was approximately 10,000 PFU per infected cell. In coinfection experiments, mutant virus packaging efficiency (COINF.) is expressed as the fold reduction in packaged mutant DNA relative to the packaged coinfecting wild-type DNA. These data were normalized to the amount of each viral DNA (mutant and wild type) present in total nuclear DNA. ND, encapsidated mutant viral DNA was below the level of accurate quantitation; NV, mutant virus was nonviable in repeated reconstruction experiments.

tions with a wild-type virus. Mutation of A repeat V (*dl*309-LS5), however, resulted in an additional 40-fold decrease in virus yield relative to the parental mutant virus in a single infection, and no detectable signal was observed in packaged virion DNA in a coinfection. This mutant virus replicated to a level comparable to that of the wild-type virus (Fig. 3), indicating that the reduction in packaged DNA was due to a *cis*-acting packaging defect. When LS mutations in A repeats IV and V were coupled (*dl*309-LS4/5), the resulting virus was nonviable. This result demonstrated that while A repeat IV lacked detectable function when A repeat V was intact, A repeat IV was essential for virus viability in the absence of A repeat V.

Additional packaging elements are located outside the originally defined packaging domain. Our previous studies demonstrated that at least two A repeats were required for virus viability (17). On the basis of this observation, the results described above with the single LS mutations in A repeats IV and V indicated that an additional packaging element(s) may lie outside the originally described packaging domain (nt 194 to 358) that may be required for virus viability in the *dl*309-194/316 mutant background. Our previous results (17) and those described below suggested that there are spatial constraints on the location and function of individual packaging elements. Therefore, a mutant virus (*dl*309-380.in6; Fig. 4) that contains a 6-bp insertion at nt 380 was constructed in a *dl*309-194/316 background. This mutant was constructed on the basis of the hypothesis that a 6-bp insertion between two critical packaging elements would significantly impair virus growth. This insertion, however, reduced packaging efficiency only three- to fourfold compared with the parental virus (Fig. 4). A similar decrease in packaging efficiency was observed when this insertion was coupled with the LS mutation in A repeat IV (*dl*309-LS4/380.in6; Fig. 4), a mutant virus in which A repeat V is critical for viability. These results suggested that if an additional packaging element(s) existed outside the nt 194 to 358 packaging domain, it likely would be located to the left of nt 380.

Two sequences which resemble A repeats by virtue of their AT-rich character are located between nt 358 and 380 (Fig. 4). One element, termed A repeat VI, is located between nt 363 and 368, and a second element, A repeat VII, is positioned between nt 370 and 375. We constructed mutant viruses which contain LS mutations in these putative packaging elements in the *dl*309-194/316 mutant virus background. The data obtained with these mutants in single infections and coinfections with a wild-type virus are shown in Fig. 4 and 5. Mutation of A repeat VI (*dl*309-LS6, nt 363 to 368) resulted in the loss of virus viability. Mutation of A repeat VII (*dl*309-LS7, nt 370 to 375) led to a three- to fivefold decrease in virus yield relative to the parental mutant. In the coinfection, the amount of packaged mutant DNA was reduced correspondingly. The double mutation of A repeats IV and VII, *dl*309-LS4/7, resulted in a comparable decrease in packaging efficiency. These results demonstrate that A repeats VI and VII constitute functional components of the Ad5 packaging signal. Since deletion of these elements in a wild-type virus background had no effect on the packaging ability of the mutant genome (22), these results lend further support to the model that the packaging signal is composed of functionally redundant elements (17, 21). From these analyses, we conclude that additional packaging ele-

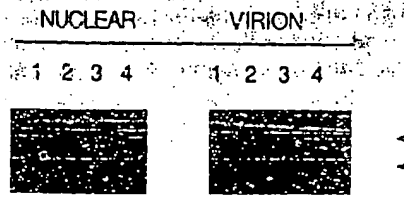

FIG. 3. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfected with the wild-type virus and mutants depicted in Fig. 2. Total nuclear DNA and virion DNA were digested with *Cla*I and analyzed by Southern hybridization, using an Ad5 left-end fragment as a ^{32}P-labeled probe. The corresponding wild-type (WT) and LS mutant (LS) left-end DNA fragments are indicated. The mutant viruses tested were *dl*309-194/316 (lanes 1), *dl*309-LS4 (lanes 2), *dl*309-LS1 (lanes 3), and *dl*309-LS5 (lanes 4).

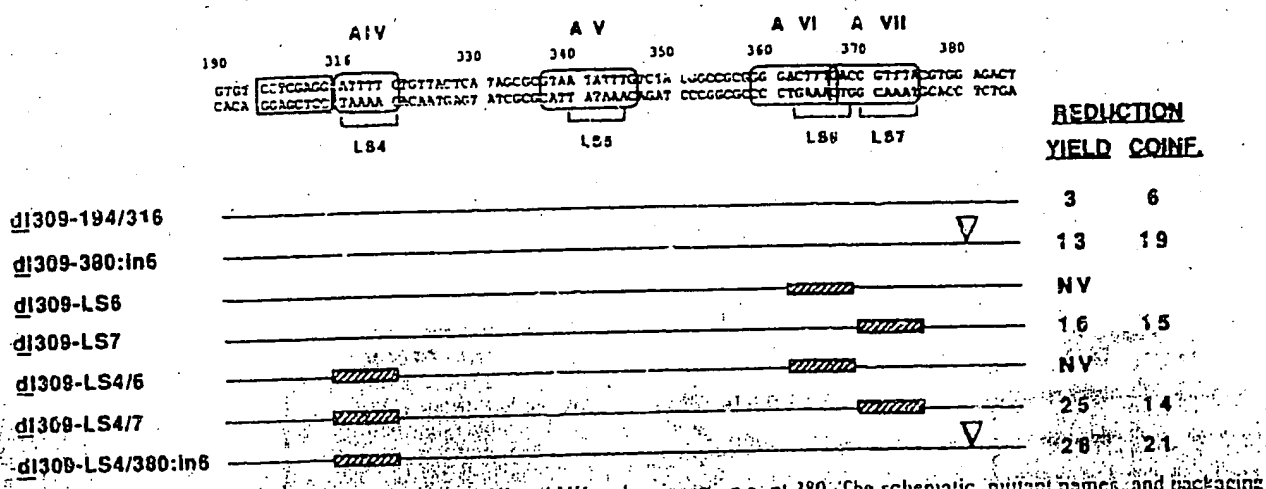

FIG. 4. Viral mutants with LS mutations in A repeats VI and VII and an insertion at nt 380. The schematic, mutant names, and packaging analyses are as described in the legend to Fig. 2. The positions and names of the LS mutants are indicated by brackets below the sequence (each LS mutation replaced the wild-type sequence with the sequence GTCGAC). The insertion mutations contains insertions of the sequence GTCGAC. The corresponding single- and double-mutant viruses are listed below; the LS mutations are indicated by hatched boxes, and the insertions are indicated by inverted triangles.

ments are located outside the originally defined boundaries of the packaging domain. In the absence of A repeats I, II, and III, A repeats V and VI play a key role in the packaging process, since inactivation of either element led to a drastic decrease in virus viability (Fig. 3, *dl*309-LS5; Fig. 5, *dl*309-LS6). A repeats IV and VII and possibly an element to the right of nt 380 help to optimize the packaging efficiency, perhaps by facilitating formation of a packaging-competent complex, but are not absolutely essential for packaging function.

A spatial requirement correlates with packaging efficiency. Our previous studies indicated that there are spacial constraints on the *cis*-acting elements involved in Ad5 packaging (17, 21). To further test the possibility that efficient assembly of a packaging-competent complex requires functional interactions between multiple packaging elements, we constructed two mutant viruses in which the spacing between a truncated packaging domain and flanking leftward and rightward sequences was altered. The parent virus used to construct these variants was a double-mutant virus, *dl*10/28 (Fig. 6) (17), that contains A repeats I, II, VI, and VII. This virus displayed a 143-fold decrease in virus yield in a single infection (17) (Fig. 6). Even though this mutant virus replicated to a level comparable to that of the wild-type virus in a coinfection, no mutant DNA was detectable in virions, indicating that the observed growth defect was largely due to a packaging deficiency of the mutant genome (17). Our previous results demonstrated that the residual packaging activity seen with this mutant is due, at least in part, to the presence of A repeats I and II (17). To test whether these two elements are sufficient to allow packaging of the mutant genome or whether functional interactions with adjacent elements are required to generate a packaging-competent substrate, two spacer mutants (*dl*10/28-194:in4 and *dl*10/28-358:in4; Fig. 6) that differ from the parental virus by a 4-bp insertion at the indicated positions (nt 194 or 358) were constructed. The results of single infections and coinfections with these mutant viruses are shown in Fig. 6.

A 4-bp insertion at the leftward deletion endpoint (*dl*10/28-194:in4) resulted in a sevenfold increase in yield in a single infection relative to the parental mutant virus. In a coinfection, the amount of packaged mutant DNA was reduced approximately 9-fold compared with the wild-type virus, while packaged DNA in the coinfection with the parental mutant was not detectable (>50-fold decrease). In contrast, the insertion of 4 bp at the rightward deletion endpoint (*dl*10/28-358:in4) resulted in the loss of virus viability. With both spacer mutants, the relationship of sequences flanking both sides of the truncated packaging domain remained unchanged with respect to each other, whereas their position relative to the intervening packaging region varied according to the site of the insertion. That these results do not reflect the phenotype of enhancer element II mutants was evident by the altered packaging efficiency in the coinfection experiment. We therefore conclude that the particular spacing of adjacent *cis*-acting elements with respect to each other is important in order to develop functional interactions between individual components of the packaging signal. In the absence of A repeats III, IV, and V, A repeats I and II function in conjunction with sequences flanking the originally defined packaging domain. In view of the results obtained with the LS mutants (Fig. 3 and 5), it is likely that A repeats VI and VII represent such interaction sites in the rightward direction. We have not yet identified the leftward sequences that participate in the formation of the packaging-specific complex. It is possible

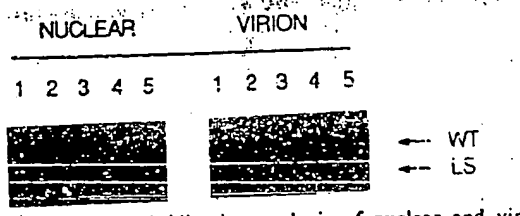

FIG. 5. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfected with the wild-type virus and mutant viruses depicted in Fig. 4. Southern hybridization analysis of total nuclear DNA and virion DNA was performed as described in the legend to Fig. 3. The mutant viruses tested were *dl*309-194/316 (lanes 1), *dl*309-LS7 (lanes 2), *dl*309-LS4/7 (lanes 3), *dl*309-380:in6 (lanes 4), and *dl*309-LS4/380:in6 (lanes 5).

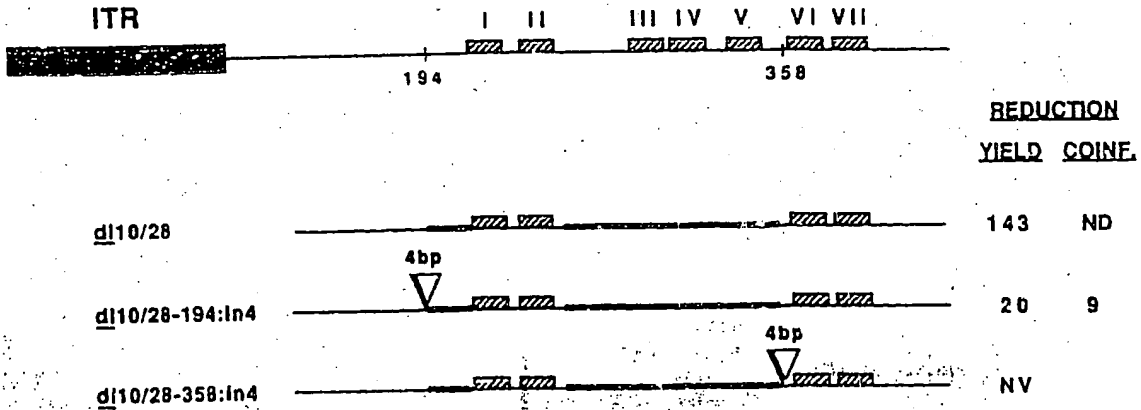

FIG. 6. Viral mutants with insertions at the leftward and rightward deletion endpoints of a minimal packaging domain. At the top are shown the positions of A repeats I through VII, depicted by shaded boxes, relative to the left terminus of the viral genome. The parental mutant virus *dl*10/28 and the spacer mutants described in the text are shown below. The deleted sequences in *dl*10/28 are indicated by solid bars; shaded boxes represent A repeats present in the mutant genome; 4-bp insertions are indicated by open triangles. The nucleotide number in each mutant virus name corresponds to the site of the 4-bp insertion. The packaging analysis of the mutant viruses was performed as described for Fig. 2.

that sequences in the ITR play a role in this process (see Discussion).

A titratable factor binds to the packaging domain. To determine whether the packaging elements represent binding sites for *trans*-acting factors, we inserted the packaging domain (nt 194 to 358; Fig. 1A) into a plasmid vector (pOR4 [9]) containing the SV40 origin of replication (pOR4-PAC$^+$). This plasmid replicates to high copy number in COS1 cells, a monkey cell line that constitutively expresses SV40 large T antigen (16). If a limiting *trans*-acting factor(s) interacts with the packaging signal in vivo, the presence of an excess of unlinked binding sites could compete for factor binding and thus prevent the formation of a functional packaging complex on the Ad5 genome. This possibility was supported by results obtained from the analysis of a number of mutant viruses described previously (17). These mutants showed a greater decrease in packaging efficiency in a coinfection with a wild-type virus relative to the reduction in yield observed in a single infection, a phenotype which could reflect a competition between the coinfecting wild-type and mutant viruses for a limiting packaging factor(s). COS1 cells were cotransfected with pOR4-PAC$^+$ plasmid DNA and wild-type *dl*309 DNA, and the infectious virus yield was determined 36 h after transfection. As controls, *dl*309 DNA was transfected alone or with the vector plasmid pOR4 lacking the packaging domain (pOR4-PAC$^-$). Additionally, total nuclear DNA was examined for the accumulation of replicated Ad5 DNA and total cellular RNA was examined for the accumulation of transcribed late viral mRNAs.

These results (Table 2) demonstrated that high levels of unlinked packaging domain sequences inhibited viral growth. Cotransfection of wild-type *dl*309 DNA with pOR4-PAC$^+$ plasmid DNA resulted in a greater than 600-fold reduction in virus yield, whereas transfection of viral DNA in the presence of an equivalent amount of vector sequences (pOR4-PAC$^-$) reduced virus yield only fivefold. Comparable levels of total viral DNA (within twofold of each other) were present in transfected cells (Fig. 7A), indicating that equal numbers of viral templates were available for packaging, and comparable levels of viral late mRNAs were observed (Fig. 7B). Therefore, the defect responsible for the reduction in virus yield must have occurred at a late step in the infectious cycle, which is consistent with a defect in the packaging of viral DNA. We conclude that the unlinked Ad5 packaging domain present in the transfected plasmid DNA likely competed for the binding of a limiting *trans*-acting component(s) required for the efficient packaging of Ad5 DNA in vivo.

DISCUSSION

In the case of bacteriophage lambda, it has clearly been shown that the process by which phage DNA is selected from a pool of intracellular DNA for packaging involves the recognition of specific viral sequences, termed *cos* sites, by a phage-encoded protein, the terminase (10, 15). Binding of the terminase to its recognition sites results in the formation of a specific nucleoprotein complex which is required for recognition and further interactions of the phage DNA molecule with the empty proheads (10, 15). Experimental evidence obtained from the analysis of several temperature-sensitive mutants suggests that Ad packages its DNA into preformed empty capsids, the proheads (2, 6–8, 12, 13, 33). The identification of packaging domains in several Ad genomes and the demonstration of polarity in the encapsidation process (3, 17, 19–21, 25, 30, 34) suggests that the specificity of the encapsidation process depends on recogni-

TABLE 2. Virus yields generated in COS1 cells transfected with *dl*309 DNA, *dl*309 DNA plus pOR4-PAC$^+$, and *dl*309 DNA plus pOR4-PAC$^-$[a]

Transfected DNA	Reduction in virus yield (fold)
*dl*309	1
*dl*309 + pOR4-PAC$^+$	>600
*dl*309 + pOR4-PAC$^-$	5

[a] Cells were transfected with the indicated DNAs, and infectious virus yields were determined by a plaque assay with cellular extracts prepared 36 h after transfection. Virus yields are expressed as fold reduction relative to the yield obtained by transfection of *dl*309 DNA alone. The total virus yield in a virus infection of COS1 cells with the wild-type virus (*dl*309) was approximately 2,000 PFU per infected cell. The infectious virus yield in a transfection with wild-type viral DNA was reduced approximately 10^4, presumably because of the inefficient nature of the transfection procedure.

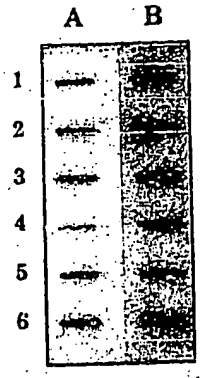

FIG. 7. Viral DNA and RNA accumulation in transfected COS1 cells. COS1 cells were transfected with *dl*309 DNA (rows 1 and 2), *dl*309 plus pOR4-PAC$^+$ (rows 3 and 4), or *dl*309 plus pOR4-PAC$^-$ (rows 5 and 6). Total nuclear DNA (lane A) and total RNA (lane B) were isolated 36 h after transfection and applied to nitrocellulose filters. Viral DNA (lane A) was probed with uniformly labeled Ad5 genomic DNA. Viral RNA (lane B) was probed with a cloned DNA fragment corresponding to late region 3.

tion of specific viral sequences present in the left end of the genome by a packaging protein(s).

In a recent report, we demonstrated that the Ad5 packaging domain is composed of five functionally redundant elements which have an additive effect on the packaging efficiency of the viral genome (17). Four of the five elements contain a repeated sequence motif, the A repeat, with the consensus sequence 5'-GT(N_{3-4})TTTG-3' (Fig. 8). On the basis of these results, we proposed three models to account for the selectivity of the packaging process. First, the individual packaging elements might represent specific binding sites for packaging proteins. Binding of a packaging protein(s) to its recognition site(s) would allow the viral genome to recognize and position itself with an empty prohead, and packaging could ensue. Second, individual packaging elements might represent an array of interspersed binding sites such that coordinate binding of the appropriate packaging proteins to all of these sites would result in the formation of a structurally defined nucleoprotein complex at the left end of the viral genome. Only molecules bearing this structure would be recognized as bona fide packaging substrates. It is possible that the formation of this complex requires further interactions with factors bound to the inverted terminal repeat sequences (ITR). This assumption is based on our previous results which demonstrated that the packaging domain (nt 194 to 358) can function independent of position and orientation but must be located close to the terminus of the viral genome in order to maintain packaging activity (21). Additional data in support of this model have been obtained from the analysis of several temperature-sensitive mutants, which suggested that factors implicated in the formation of the DNA synthesis initiation complex may play a role in viral DNA encapsidation (5). Third, as a consequence of their AT-rich character, the A repeats could also represent functional bending loci (35). This model implies that the packaging specificity is determined by a structural rather than a sequence-specific feature of the viral genome. It is conceivable that a packaging protein recognizes the overall conformation of a DNA molecule repre-

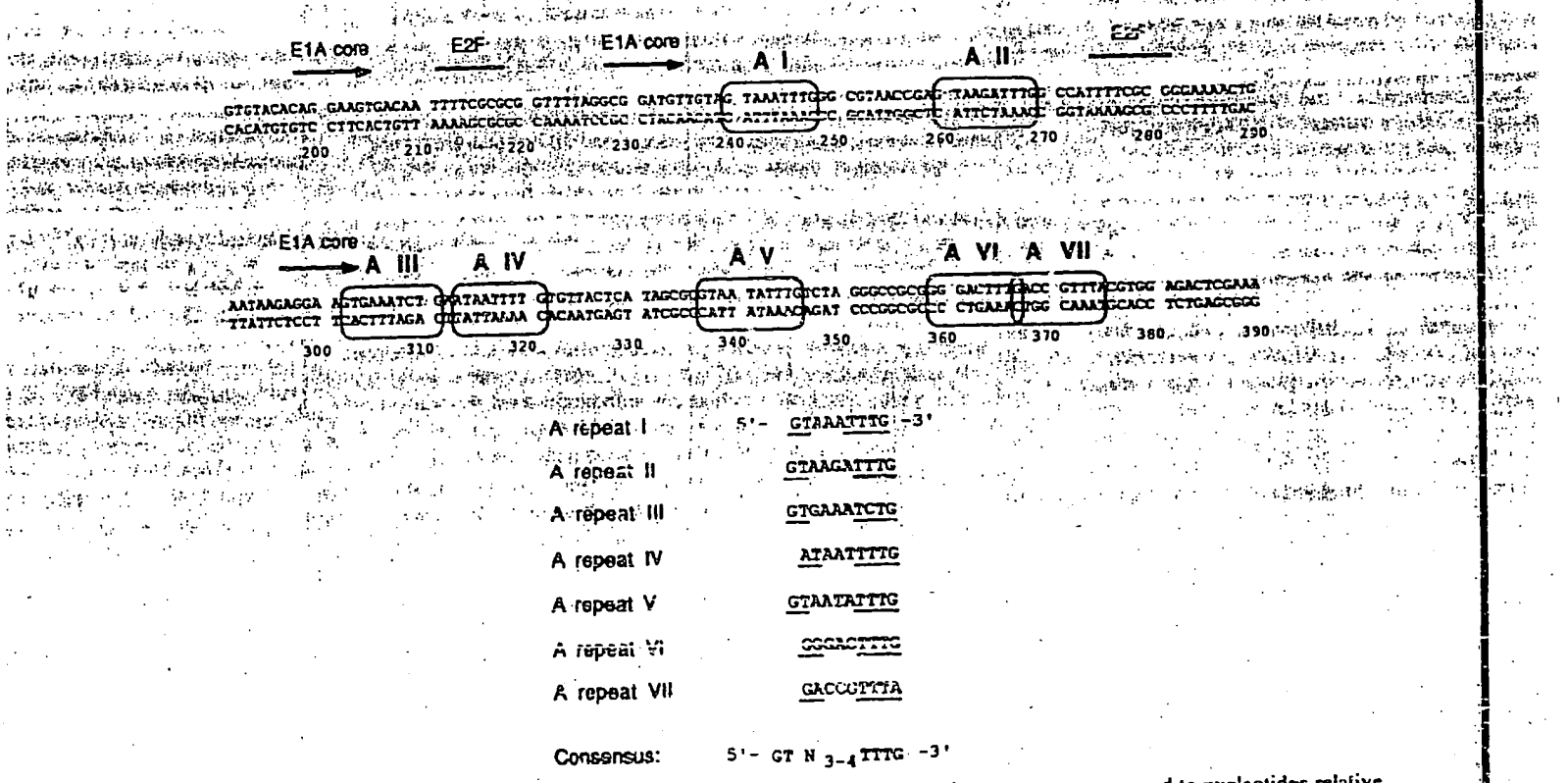

FIG. 8. Nucleotide sequence of the Ad5 genome between nt 191 and 390. Numbers below the sequence correspond to nucleotides relative to the left terminus. A repeats I through VII are encircled. Binding sites in this region for transcription factors are shown above the sequence (E1A core enhancer element I shown by arrows and E2F binding sites shown by dark bars) (1, 23, 26). The nucleotide sequences of the individual A repeats are shown below, along with a consensus sequence drawn from a comparison of these sites.

sented as bent DNA or that this structure is required for recognition of a prohead. To examine the possibility that the A repeats represent functional bending sites, we analyzed the electrophoretic migration of restriction fragments spanning the various LS mutations relative to the electrophoretic mobility displayed by the corresponding parental restriction fragment. While the left end of the viral genome displayed aberrant electrophoretic mobility consistent with bent DNA (data not shown; 11, 29) and particular LS mutations altered the mobility of mutant DNA fragments, our analysis did not reveal any obvious correlation between the packaging efficiency of a mutant virus and the molecular conformation of its mutant genome, determined as bent DNA (data not shown).

In this report, we provide further evidence for the proposal that the A repeats constitute functionally redundant packaging elements. Specific mutation of A repeat V, in the absence of A repeats I, II, and III (Fig. 2), greatly reduced the packaging efficiency of the mutant genome (Fig. 3), clearly demonstrating a functional involvement of this repeat in the packaging process. Analysis of additional LS mutant viruses has shown that additional elements (A repeats VI and VII; Fig. 4) are required for efficient packaging of the viral genome. These elements correspond to sequences which strongly resemble the A repeat consensus sequence (Fig. 8). The fact that inactivation of individual repeats impaired the packaging efficiency of the mutant viruses to different extents (compare *dl*309-LS5, *dl*309-LS4, *dl*309-LS6, and *dl*309-LS7 in Fig. 2 and 4) is indicative of a functional hierarchy between individual members of the A repeat family. The different activities displayed by the packaging elements might reflect differences in affinity of the individual binding sites for factor binding. In the absence of A repeats I, II, and III, A repeats V and VI play a key role in the packaging process, since inactivation of either element had a drastic effect on virus viability (Fig. 2 and 4). In the presence of A repeats V and VI, the remaining elements, A repeats IV and VII and possibly an element to the right of nt 380, were not absolutely required for viral growth but helped to optimize the packaging efficiency of the mutant genome (Fig. 4).

From the results obtained from the analysis of spacer mutants (*dl*10/28-194:in4 and *dl*10/28-358:in4; Fig. 6), we propose that a particular spacing of adjacent A repeat motifs with respect to each other is required to promote efficient packaging. This likely reflects the need for the coordinate binding of packaging proteins to multiple sites. In the absence of A repeats IV and V, A repeats I and II are required but not sufficient to allow packaging of the mutant genome (Fig. 6). The fact that the introduction of approximately one half of a helical turn at the rightward border of the deletion (*dl*10/28-358:in4) resulted in the loss of virus viability suggests that the residual packaging efficiency seen with *dl*10/28 requires functional interactions between A repeats I and II and additional elements located rightward of nt 358. In view of the results obtained with the LS mutants described above, it is likely that these elements are represented by A repeats VI and VII. In this context, it is interesting to note that the most highly conserved tetranucleotide (5'-TTTG-3') portion of the A repeat consensus sequence is separated by exactly two turns of the DNA helix (21 bp) between A repeats I and II and between A repeats V and VI, the most important repeats based on functional assays (described above and in reference 17). The spatial constraint observed between the packaging elements strongly suggests that protein-protein interactions are important for the generation of a functional packaging complex. It is possible that stable binding of a protein to its recognition sites requires cooperation between proteins bound to different sites, or as proposed in the second model, the simultaneous binding of proteins to multiple sites is required for the formation of a defined packaging-specific nucleoprotein complex. Alteration of the spatial relationship between A repeats I and II and leftward flanking sequences improved viral growth (*dl*10/28-194:in4; Fig. 6). As was evident in the coinfection experiment, this increase was due to an increased packaging efficiency of the mutant genome. We have not identified the leftward sequences that participate in the packaging process, but it is conceivable that this complex involves interactions between factors bound to the left-terminal ITR and proteins bound to the packaging domain.

In our previous analysis of Ad5 packaging mutants, we noted that a number of mutants showed a greater decrease in packaging efficiency in coinfection experiments than was expected from results obtained with the same mutant viruses in a single infection (17). We speculated that the greater reduction observed in the coinfection with these mutants represented a competition between the wild-type and mutant genomes for limiting concentrations of a packaging protein(s). Here we provide further evidence that suggests that the A repeats represent sequence-specific binding sites for a *trans*-acting titratable factor(s). In the cotransfection experiment, the presence of an excess of unlinked packaging domain sequences efficiently interfered with normal viral growth (Table 2). Since viral DNA replication and late gene transcription were unaffected, we believe that the reduction in viral yield reflects a successful competition of the packaging sequences contained in the plasmid with those of the viral genome for a limiting *trans*-acting factor required for packaging of the viral DNA molecule in vivo. These results also demonstrated that the originally defined packaging sequences, encompassing nt 194 to 358, are sufficient for factor binding. However, they do not exclude the possibility that additional protein-protein interactions are required to create a packaging-competent viral DNA substrate.

In conclusion, our observations support the model which proposes that the *cis*-acting packaging elements represent recognition sites for proteins involved in the packaging process and that specific protein-DNA interactions provide the molecular basis for the observed packaging specificity. The generation of the specialized nucleoprotein structure required for the selective encapsidation of the Ad genome appears to require multiple DNA-protein and protein-protein interactions.

ACKNOWLEDGMENTS

We thank the members of our laboratory, Carl Anderson, and Nick Muzyczka for many stimulating discussions and Tina Philipsberg for excellent technical help.

This research was supported by Public Health Service grant CA28146 from the National Cancer Institute.

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JOURNAL OF VIROLOGY, May 1997, p. 3375–3384
0022-538X/97/$04.00+0

Vol. 71, No. 5

Bipartite Structure and Functional Independence of Adenovirus Type 5 Packaging Elements

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Received 14 November 1996/Accepted 24 January 1997

Selectivity and polarity of adenovirus type 5 DNA packaging are believed to be directed by an interaction of putative packaging factors with the *cis*-acting adenovirus packaging domain located within the genomic left end (nucleotides 194 to 380). In previous studies, this packaging domain was mutationally dissected into at least seven functional elements called A repeats. These elements, albeit redundant in function, exhibit differences in the ability to support viral packaging, with elements I, II, V, and VI as the most critical repeats. Viral packaging was shown to be sensitive to spatial changes between individual A repeats. To study the importance of spatial constraints in more detail, we performed site-directed mutagenesis of the 21-bp linker regions separating A repeats I and II, as well as A repeats V and VI. The results of our mutational analysis reveal previously unrecognized sequences that are critical for DNA encapsidation in vivo. On the basis of these results, we present a more complex consensus motif for the adenovirus packaging elements which is bipartite in structure. DNA encapsidation is compromised by changes in spacing between the two conserved parts of the consensus motif, rather than between different A repeats. Genetic evidence implicating packaging elements as independent units in viral DNA packaging is derived from the selection of revertants from a packaging-deficient adenovirus: multimerization of packaging repeats is sufficient for the evolution of packaging-competent viruses. Finally, we identify minimally sized segments of the adenovirus packaging domain that can confer viability and packaging activity to viruses carrying gross truncations within their left-end sequences. Coinfection experiments using the revertant as well as the minimal-packaging-domain mutant viruses strengthen existing arguments for the involvement of limiting, *trans*-acting components in viral DNA packaging.

Increasing the usefulness of adenovirus as a vector for the delivery of foreign genes into mammalian cells calls for the development of an in vitro packaging system to assemble recombinant viruses carrying a packaging signal fused to heterologous sequences. Such a packaging system could entail a number of advantages. First, the size of the foreign gene, which is restricted to about 10 kbp in order to propagate the virus in tissue culture (3), could be expanded substantially. Second, with all viral genes absent, the overwhelming immune response of the host to adenovirus infection potentially could be minimized. This response, both humoral and cytotoxic T-cell mediated, has mainly been ascribed to various viral gene products produced even in the context of replication-deficient second-generation adenovirus vectors (12, 32, 33). Little information is presently available about the basic mechanism of encapsidation of adenovirus DNA.

The assembly of adenovirus particles has been studied extensively by using a large number of viral temperature-sensitive mutants blocked at different stages of assembly at the restrictive temperature and by pulse-chase kinetic analyses (6–8, 10, 11). These early studies clearly established that adenovirus DNA is inserted into preformed, empty capsids late in the viral life cycle. Whether viral genomes enter the prohead in association with core proteins or as a separate entity is unclear, as is the exact structure of the viral DNA before and during entry. *cis*-acting packaging sequences in the adenovirus genome are required to direct selective encapsidation from the left end of the viral DNA. Polarity of adenovirus DNA packaging was initially demonstrated in studies on viral incomplete particles containing viral DNA molecules of subgenomic length (5, 31), where a striking overrepresentation of left-end sequences was revealed, suggesting that DNA packaging occurs in a polar fashion from left to right. It was subsequently shown for adenovirus type 16 (Ad16) and Ad3 that a *cis*-acting packaging domain is located within the left 390 bp (19, 29). Sequence alignments of Ad3 with Ad5 and Ad12, representatives of adenovirus subgroups A, B, and C, revealed a large degree of sequence conservation within the interval between nucleotides (nt) 237 and 491 (25). This conservation is likely due to a conserved packaging signal in addition to a number of transcription factor binding sites located within this region. Conservation of the packaging sequences may reflect the dependence of all adenoviruses on a *cis*-acting encapsidation signal, suggestive of a similar mechanism of selective and polar DNA packaging for all adenovirus subgroups.

The *cis*-acting packaging domain in Ad5 is located in the left end 380 bp (16, 17, 20) and overlaps two distinct enhancer elements (Fig. 1A). Enhancer element I consists of a repeated sequence motif (21) and stimulates E1A transcription specifically upon binding of a cellular nuclear factor, EF-1A (4). Mutations in element I affecting its function can be efficiently complemented by propagation of the virus in 293 cells, a cell line that constitutively expresses the viral E1A and E1B gene products (18). Element II enhances transcription in *cis* from all early transcription units by an unknown mechanism (22). Element II mutations result in a decrease in early transcription and, since some of the early gene products are required for DNA replication, in a corresponding reduction in virus growth. This *cis*-acting defect can be efficiently complemented in *trans* by providing all of the early gene products in a mixed infection with wild-type virus (22). Deletion of the Ad5 packaging do-

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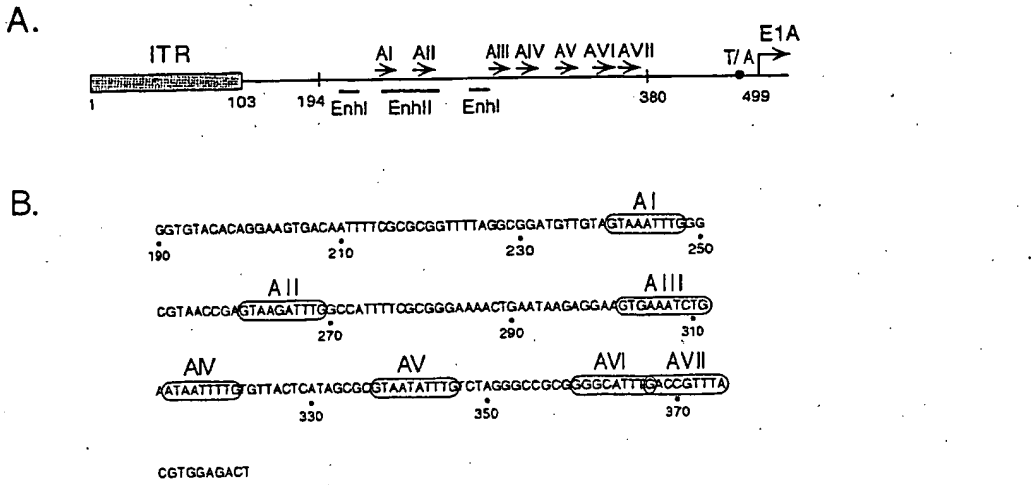


FIG. 1. (A) The Ad5 genomic left end and packaging domain. The schematic diagram shows the ITR, the packaging/enhancer region (nt 194 to 380), and the E1A 5′ flanking region. Numbers indicate nucleotide positions relative to the left-end terminus. The packaging repeats (AI through AVII) are represented by arrows. The ITR is represented by a shaded box, and the E1A transcriptional start site is indicated by an arrow at nt 499. Transcriptional elements include a TATA box motif (T/A; black circle) and enhancer elements I and II (EnhI and EnhII; indicated by lines). (B) Nucleotide sequence of the Ad5 packaging domain. Numbers correspond to nucleotides relative to the left-end terminus. AI through AVII are circled.

main resulted in nonviability, but wild-type growth could be restored by the substitution of the left-end 353 bp at the right end of the genome (21). The Ad5 packaging domain was shown to be flexible with respect to its position as well as orientation within certain boundaries. However, it must be located within 600 bp of the inverted terminal repeat (ITR) (20). Whether ITR sequences themselves are required for adenovirus DNA packaging has not been determined yet due to a *cis* requirement for DNA replication.

Detailed analysis of the Ad5 packaging domain by deletion as well as linker scanning mutagenesis (16, 17) revealed that it consists of at least seven functional units called A repeats due to their AT-rich character (Fig. 1B). These elements are functionally redundant, but in spite of their redundancy, they follow a hierarchy of importance, with elements I, II, V, and VI as the functionally most dominant repeats. In support of a model implicating the A repeats as binding sites for limiting, *trans*-acting packaging components, cotransfection of an excess of packaging-domain sequences and wild-type adenovirus genomes resulted in a dramatic decrease in virus yield while DNA replication as well as late transcription were unaffected (17). This result is thought to reflect a competition event ongoing between unlinked packaging sequences and the viral genomes for the recognition of limiting packaging components. Consistent with this model is the fact that there are spatial constraints between individual packaging elements (17), presumably because the protein components involved have to interact with the packaging elements in a coordinate fashion. Notably, elements I and II as well as elements V and VI are separated by a spacing of 21 bp, or two helical turns of the DNA. Factors bound to these repeats would come to locate to the same side of the DNA double helix to interact with each other and/or components outside the packaging domain to mediate recognition of the prohead followed by DNA encapsidation.

Here we focus on an analysis of the 21-bp region separating A repeats I and II (AI and AII) and A repeats V and VI (AV and AVI). Mutagenesis within this region identifies additional sequence determinants as part of the packaging consensus motif. Of special importance is the presence of a CG dinucleotide located downstream of each of the four A repeats. Spacing between the consensus 5′-TTTG-3′ and the CG located downstream, rather than spacing between individual A repeats, appears critical. We propose an extended consensus motif for the packaging elements which could represent a binding site for one or several packaging factors. In addition, we define minimal packaging sequences that exhibit maximal packaging activity in functional assays when introduced into a packaging domain-minus background. These minimal packaging domains may prove useful for the construction of new adenoviruses in the field of gene therapy. Finally, we selected revertants from a packaging-deficient adenovirus and show that in each case, A repeats were amplified, providing genetic evidence that these elements act as independent units in viral DNA packaging. The revertants exhibit a phenotype in which viruses containing more A repeats have a competitive advantage over viruses with fewer A repeats in coinfections with either wild-type virus or each other. These results again support an argument for the existence of limiting protein components as part of the packaging machinery.

MATERIALS AND METHODS

Plasmids and virus constructions. Ad5 *dl*309, the parent for all viruses described in this report, is a phenotypically wild-type virus that contains a unique *Xba*I cleavage site at 3.8 map units (24). *dl*309-194/316 and *dl*309-274/358, described previously (16, 17), carry deletions of sequences between nt 194 and 316 and nt 274 and 358, respectively. *dl*309-274/376 carries a deletion between nt 274 and 376. Mutations were constructed originally in plasmids pKS-194/316 and pKS-274/358, which contain the left-end *Xba*I fragment (nt 1 to 1339) of each mutant virus cloned into the polylinker region of the pBS-KS (+) Bluescript vector (Stratagene). They were used as parents for the generation of a series of linker scanning and insertion mutations by the method of Kunkel (26). Plasmid pBR-53/322 comprises the left-end Ad5 *Xba*I fragment containing a deletion between nt 53 and 322 cloned into pBR322. It served as a parent for further deletion of sequences between nt 640 and 814 as well as nt 455 and 814, using *Rsa*I/*Nae*I and *Pvu*II/*Nae*I restriction digestion, respectively. pBR-194/814 and pBR-53/814 have sequences between nt 194 and 814 and nt 53 and 814 deleted. A monomer and dimer of viral sequences located between nt 334 and 385 which contain AV, AVI, and AVII was cloned into the 194/814 deletion. A dimer of the nt 334 to 385 fragment as well as 12 head-to-tail copies of an oligonucleotide containing AVI ((5′-TCGACCGCGGGGACTTTGACC-3′:5′-TCGAGGT CAAAGTCCCCGCGG-3′) were cloned into the 53/814 deletion in either orientation. All mutations were verified by nucleotide sequence analysis. The mutations were subsequently rebuilt into intact viruses by the method of Stow (30).

Virus was amplified and titers were determined on 293 cells. Mutant viruses were screened by restriction analysis of viral DNA obtained from infected 293 cells by the Hirt procedure (23). The authenticity of all linker scanning mutations was confirmed by amplification of viral left-end sequences spanning nt 1 to 1347 by PCR; the left-end fragments were gel purified and used as templates for the Double Stranded DNA Cycle Sequencing System (GIBCO BRL).

Cell lines and infections. 293 cells (18) and A549 cells, a human lung epithelium cell line, were maintained as monolayers in Dulbecco modified Eagle medium containing 10% bovine calf serum (HyClone). Virus stocks were generated by three freeze-thaw cycles of infected cell lysates, and titers were determined by plaque assays on 293 cells. Alternatively, virus particles were purified by CsCl equilibrium gradient sedimentation and titers were determined by optical density, whereby 1 optical density unit at A_{260} equals 10^{12} particles. Virus infections were performed at a multiplicity of infection of 3 PFU per cell or 200 particles per cell for 1 h at 37°C. Cells were then washed twice with Tris-buffered saline solution and overlaid with fresh medium.

Selection of revertants of a packaging-deficient adenovirus. The parent virus for the selection of revertants was constructed as follows. Plasmid pBR-194/455+AVI^{6}ENH$^-$ contains the Ad5 left-end *Xba* fragment carrying a deletion between nt 194 and 455, with six copies of an oligonucleotide containing AVI (see above) introduced at the deletion junction. Insertion of the AVI head-to-tail multimer created an *Xho* site at the right junction, which was used for the insertion of four copies of the E1A core enhancer element binding sites to ensure efficient E1A expression (2, 4). An *Eco*RI site separates the hexamer of AVI from the E1A core tetramer in the resulting plasmid, pBR-194/455+AVI^{6}ENH$^+$. The construct was then rebuilt into a *dl*309 virus background. The resulting virus had acquired two additional A repeats during the first growth cycle (see Results). To avoid recombination events between adenovirus left-end sequences integrated in the genome of 293 cells and parental genomes, the virus was subsequently propagated on A549 cells after initial recovery of plaques from 293 cells. After 12 passages of a number of independent isolates, an accelerated onset of cytopathic effect was evident. Putative revertants were plaque purified and amplified, and the revertant phenotype of each of the viruses relative to the parent was confirmed in single-infection and coinfection experiments. Left-end sequences of parent and revertant viral DNAs were compared by restriction and nucleotide sequence analysis as described above.

Determination of virus yield and packaging efficiency. To determine virus yield in a single infection, infected cell lysates were prepared 48 h postinfection, and the amount of infectious virus was determined by plaque assays on 293 cells. Packaging efficiency of the mutant viruses was determined in a coinfection of 293 cells with both mutant and wild-type *dl*309 virus. Forty-eight hours postinfection, one half of the cells was used to isolate total nuclear DNA; the other half was used for the preparation of viral DNA from purified virions as previously described (17). Briefly, total nuclear DNA was isolated from infected cells following the addition of Nonidet P-40 to 0.6% and precipitation of the nuclei by centrifugation. Viral DNA was prepared after lysis of infected cells in 0.2% deoxycholate and 10% ethanol at pH 9. Following treatment with RNase A and DNase, virions were lysed in 0.5% sarcosyl, and viral DNA was digested with proteinase K, phenol-chloroform extracted, and precipitated with ethanol. Both DNA preparations were digested with *Xba*I (*Xba*I and *Eco*RI for the revertant viruses and their parent) to distinguish between mutant and wild-type DNAs and quantitated by Southern blot hybridization (27) using pE1A-WT (21), ^{32}P labeled by the random primer method (13), as a probe. The relative intensities of the bands in autoradiograms were determined by densitometric scanning, using blots that were exposed to X-ray film without an intensifying screen. Quantitation of the data was performed by using the public domain NIH Image program (written by Wayne Rasband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part no. PB93-504868). The data presented for virus yield in the single infections and the data for packaging efficiency based on coinfection experiments represent the averages of three to five independent experiments.

RESULTS

Spatial and sequence determinants important for viral DNA packaging in the spacer region between A repeats. As previously reported, changes of spacing between different A repeats, as well as between A repeats and regions outside the packaging domain, can have dramatic effects on viral packaging efficiency (16, 17). In this study, we focused on potential spacing requirements as well as sequence requirements within the two 21-bp spacer regions separating AI and AII, and AV and AVI. These four packaging elements have previously been implicated as the most relevant A repeats within the functional hierarchy of packaging elements (17). It was hypothesized that the separation of AI from AII and of AV from AVI by 21 bp, or two turns of the DNA double helix, may reflect a requirement for an interaction of *trans*-acting packaging components with packaging repeats on the same face of the DNA.

We constructed a panel of mutant viruses carrying linker scanning and insertion mutations within the 21-bp spacer regions between AI and AII as well as between AV and AVI. In view of the functional redundancy of the A repeats, we introduced all mutations between AI and AII in the context of a deletion of AIII, AIV, and AV (*dl*309-274/358). Mutations in the spacer region between AV and AVI were constructed in a mutant background with a deletion of AI, AII, and AIII (*dl*309-194/316). We tested all recombinant viruses in two independent assays. In the first assay, infectious virus yield was determined by plaque assays after a 48-h single infection of 293 cells. The adenovirus DNA packaging domain overlaps an enhancer element (element II) which stimulates transcription from all viral early transcription units in *cis* by an unknown mechanism. Mutations affecting the function of this element result in a decrease in viral DNA replication and consequently in a reduction of overall viral growth (22). This reduction (element II phenotype) can be complemented in a coinfection with wild-type virus providing all viral gene products in *trans*. To determine what portion of the reduction in overall growth as observed in the single infection is caused by a packaging defect, we based our second assay on a coinfection of 293 cells with the mutant and wild-type viruses. Total replicated DNA and packaged DNA were isolated from 293 cells after a 48-h infection. Mutant and wild-type DNAs were distinguished by restriction digestion, and their relative amounts were quantitated by Southern blot analysis. That way, the amount of packaged mutant virus DNA relative to the coinfecting wild-type DNA could be normalized to the levels of replicated DNA of each mutant and wild-type virus. This quantitation of the data enabled us to determine the reduction of viral DNA packaging independent of an element II phenotype whereby the coinfecting wild-type virus served as an internal control.

Figure 2A shows the mutations introduced into the spacer between AI and AII and summarizes the results obtained with these mutants. Figure 2C shows a Southern blot of a representative coinfection experiment. The parent virus, *dl*309-274/358, displayed a sixfold reduction in a single infection and a twofold decrease in packaging efficiency in a coinfection. A 4-bp insertion between AI and AII (IN4 251/252) resulted in a dramatic decrease in virus growth in a single infection, and packaged mutant viral DNA was not detectable in the coinfection. A 3-bp substitution overlapping the site of this insertion (LS 250–252) had no effect on virus growth compared to the parent virus. Mutant LS 255–260, carrying a 6-bp linker scanning mutation within the spacer, was dramatically defective in the single-infection and coinfection experiments. This result could reflect the fact that the first two nucleotides (GT) of the AII consensus sequence (17) were mutated; alternatively, it could implicate previously unrecognized sequences as part of the *cis*-acting packaging consensus motif. To distinguish between the two possibilities, 3-bp substitutions were introduced across the region between nt 249 to 261. Mutants LS 250–252, LS 253–255, and LS 259–261 grew and were packaged at the levels of the parent virus, whereas LS 256–258 displayed a 68-fold reduction in growth and packaged DNA was not detectable in a coinfection. This finding suggested that the defect observed with the mutant virus LS 255–260 was due to mutation of the three nucleotides CCG. The dinucleotide CG within this motif is located 11 nt downstream of the central thymidine in the thymidine triplet of AI. A CG dinucleotide is conserved in the same position relative to AI, AII, AV, and AVI (Fig. 1B), indicating that it may function in conjunction with the previously defined consensus packaging motif. The mutant virus LS

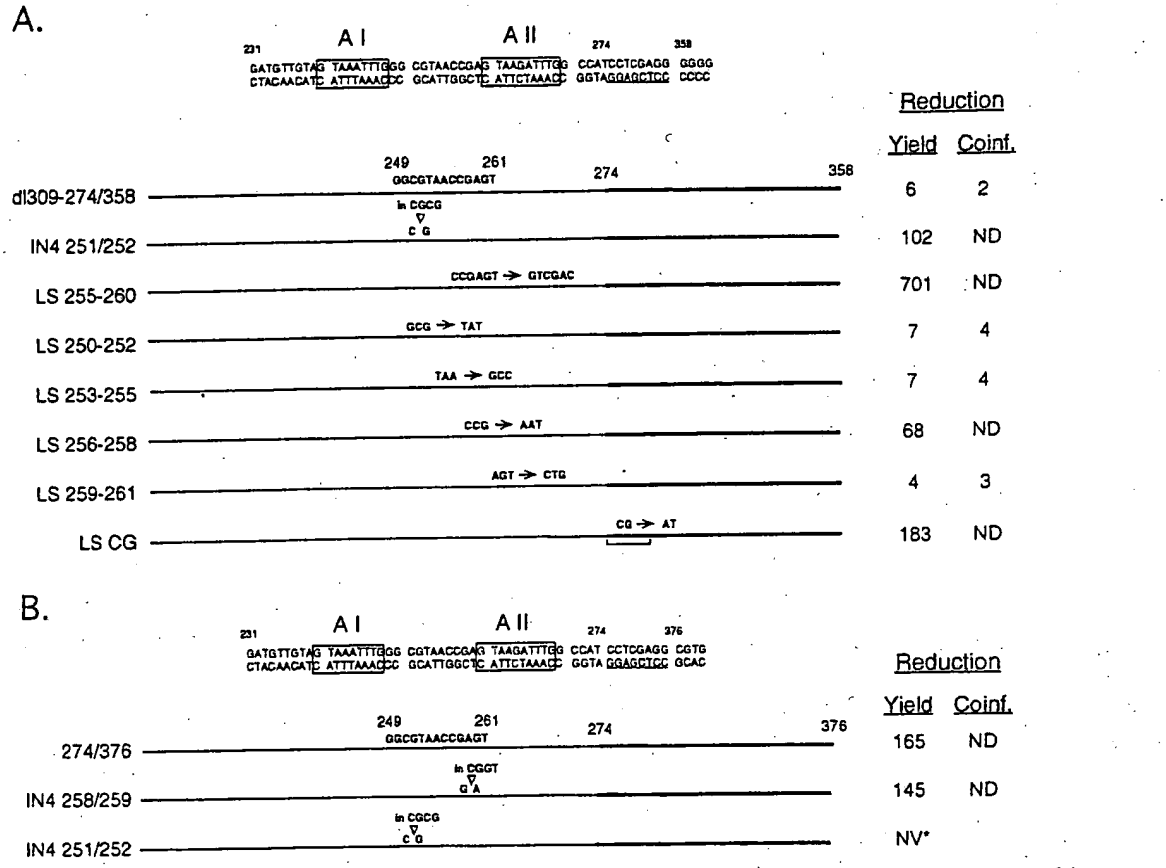


FIG. 2. Mutations surrounding AI and AII. (A) The sequence of the parent virus *dl*309-274/358 between nt 231 and 361 is shown at the top. AI and AII are boxed; an *Xho* linker present at the 274/358 deletion junction is underlined. A schematic of the *dl*309-274/358 deletion is shown just below, with the deletion indicated by a dark bar (nt 275 to 357) and the sequences between nt 249 to 261 indicated. The mutant viruses described in the text are depicted underneath and are named according to the nucleotides mutated. The site of a 4-bp insertion or the nucleotides mutated are shown below the parent virus nucleotide sequence, with the corresponding nucleotide changes indicated. All mutant viruses except for LS CG carry mutations within the region between nt 249 and 261. Mutant LS CG has a mutation in the CG sequence in the *Xho* linker (represented by a bracket). Mutant virus yields in the single infections are expressed as fold reduction in yield relative to that of the wild-type virus. The results from the coinfection experiments and Southern blot analysis (Coinf.) are expressed as fold reduction in packaged mutant DNA relative to packaged wild-type DNA. These data were normalized to the levels of viral DNA (mutant and wild type) present in total nuclear DNA. ND, packaged viral DNA was below the level of accurate quantitation. (B) Viral mutants with 4-bp insertions in the spacer region between AI and AII in the background of the parent virus 274/376. The nomenclature and results of viral infection are as described for panel A. NV*, transfection experiments yielded plaques which could not be amplified without reversion to wild-type virus. (C) Southern blot analysis of viral DNA represented either in total nuclear DNA or in virion particles isolated from 293 cells coinfected with wild-type virus and the mutant viruses depicted in panel A. Total nuclear DNA or virion DNA was digested with *Xba*I and subjected to Southern blot analysis using an Ad5 left-end fragment as a ^{32}P-labeled probe. The corresponding left-end fragments of mutant (M) and wild-type (WT) genomes are indicated on the left. The mutant viruses tested were *dl*309-274/358 (lanes 1), LS 250–252 (lanes 2), LS 253–255 (lanes 3), LS 256–258 (lanes 4), LS 259–261 (lanes 5), LS 255–260 (lanes 6), IN4 251/252 (lanes 7), and LS CG (lanes 8).

CG emphasizes the importance of the CG located downstream of AII. During the construction of the parent virus *dl*309-274/358, the CG dinucleotide downstream of AII had originally been deleted but was coincidentally replaced by an *Xho* linker (16). Transversion of the dinucleotide in the mutant LS CG resulted in a dramatically defective phenotype of the mutant virus in single-infection and coinfection experiments, suggesting that even in the context of nonviral sequences, the presence of the dinucleotide CG downstream of AII is critical for viral DNA packaging.

Insertion of four nucleotides between AI and AII in mutant IN4 251/252 dramatically affected viral growth and packaging efficiency. This mutation, however, alters both spacing between AI and AII and the spacing between AI and the CG dinucleotide downstream of it. To distinguish between these two possibilities, we rebuilt IN4 251/252 as well as a 4-bp mutation to

the right of the CG dinucleotide, IN4 258/259, into a mutant virus background lacking AIII, AIV, AV, AVI, and AVII (*dl*309-274/376 [Fig. 2B]). This mutant virus background was selected to exclude the possibility that effects on viral DNA packaging are not due to changes of spacing between AI and AII but instead are due to changes of spacing between AI and the remaining repeats to the right of AII (AVI and AVII) present in the *dl*309-274/358 parent virus. The results of single and coinfections with these viruses are displayed in Fig. 2B. The parent virus *dl*309-274/376 is 165-fold reduced in the single infection, and packaged mutant virus DNA is not detectable in the coinfection due to the large truncation of the packaging domain. Mutant 274/376:IN4 258/259 displayed a very similar phenotype, whereas mutant 274/376:IN4 251/252 was nonviable. These data emphasize that rather than a 21-bp spacing between AI and AII, the spacing between AI and the CG dinucleotide located 11 bp to the right appear to be critical for viral DNA packaging.

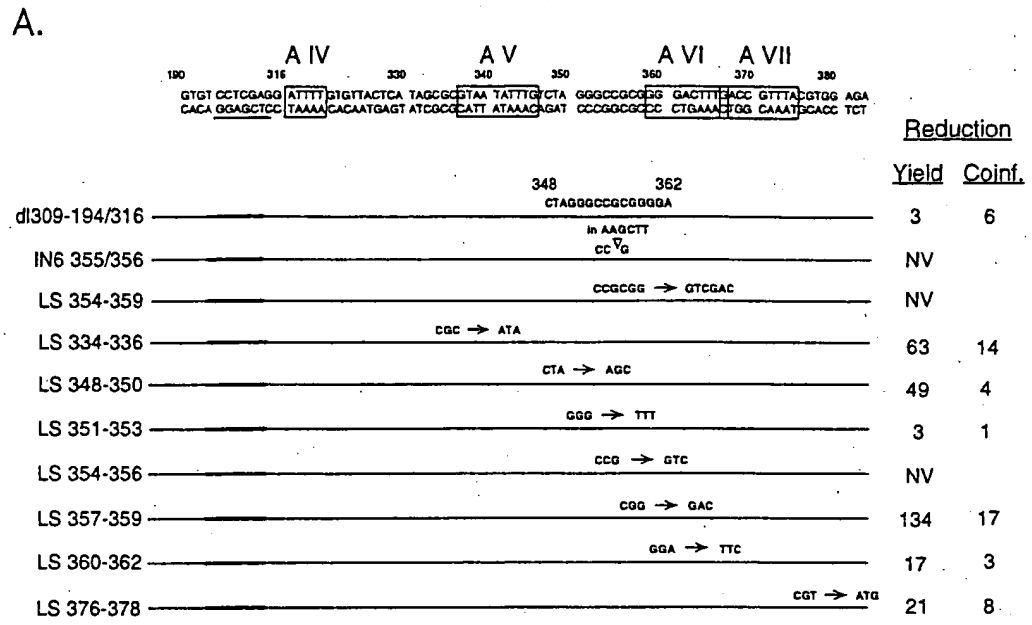
We introduced a similar set of mutations into the spacer region between AV and AVI in the context of a mutant virus that carries a deletion of AI through AIII (*dl*309-194/316). The results are summarized in Fig. 3A, and a representative Southern blot of a coinfection experiment is shown in Fig. 3B. The parent virus was reduced threefold in overall growth in a single infection, and its packaging efficiency was sixfold less than that of the coinfecting wild-type virus. Insertion of 6 nt into the CG located 11 bp to the right of AV resulted in a nonviable virus (IN6 355/356). Two mutant viruses, LS 354–359 and LS 354–356, with 6- and 3-bp substitutions overlapping the CG dinucleotide, respectively, also were nonviable. Their defective phenotypes implicate the CG dinucleotide downstream of AV as an important determinant for adenovirus DNA packaging in accordance with the previous set of mutants. In contrast to the previous results, however; mutation of the CG dinucleotide downstream of AVI (mutant LS 376–378) did not significantly affect viral packaging efficiency; overall growth in the single infection was reduced 21-fold, but virus growth in a coinfection was not affected. Most of the remaining 3-bp substitution mutants displayed a phenotype that is reminiscent of the element II phenotype described above. That is, defects observed in a single infection could be fully or largely complemented in a coinfection with a wild-type virus. The two most dramatic examples of this phenotype were caused by the mutations in LS 348–350 and LS 357–359. These mutants exhibited a small-plaque phenotype, which could, at least partially, account for the incongruities between the data from the single-infection and coinfection experiments. In spite of the generally more complex phenotypes observed with this panel of mutants, the presence of the dinucleotide CG downstream of AV appears to be necessary for viral DNA packaging as shown above for AI and AII. We suggest an extended consensus packaging motif for AI, AII, and AV, which in addition to the previously defined AT-rich motif contains a dinucleotide CG in position 11 and 12 downstream of the central thymidine of the invariable thymidine triplet (Fig. 4). This dinucleotide is also conserved downstream of AVI, but our mutational studies do not confirm its significance with respect to viral DNA packaging.

Defining minimal adenovirus packaging domains with maximal function in vivo. The adenovirus packaging repeats are functionally redundant, and in addition to the seven known A repeats, the presence of other packaging elements located outside the previously defined packaging domain has been suggested (17). For these reasons, it has been difficult to define a packaging domain of minimal size which is necessary and sufficient for adenovirus DNA packaging in vivo. We began our search for such a minimal packaging domain by deleting regions flanking the packaging domain in the context of a mutant virus 53/322, which carries only AV, AVI, and AVII. Figure 5 shows the parent virus 53/322 and two viruses derived from it, 53/322:640/814 and 53/322:455/814, which combine the 53/322 deletion with deletions extending from nt 640 to 814 and from nt 455 to 814, respectively. None of these viruses show significantly reduced abilities to package their DNA in a coinfection with wild-type virus. We conclude from these data that the regions between nt 53 to 322 and between nt 455 and 814 do not contain sequences with a critical function for viral DNA packaging in the context of a packaging domain that consists of AV, AVI, and AVII.

It has previously been demonstrated that the insertion of multimeric A repeats can rescue the nonviable phenotype of a virus lacking AI through AV (17). In a similar approach, we used a deletion of the entire packaging domain between nt 194 and 814 for the reinsertion of sequences that may function as minimal packaging domains (Fig. 5). The nt 814 position was chosen since previous results demonstrated that the Ad5 packaging sequences must be positioned within 600 bp of the left terminus (20). Thus functional packaging elements to the right of nt 800 appeared unlikely. Based on the results just described, we first introduced a monomer and dimer of a fragment containing AV, AVI, and AVII, which we will refer to as A(V-VII) and A(V-VII)^2, respectively. The fragments were inserted in both orientations. The parent virus 194/814 was nonviable, but viability could be restored by a monomeric and dimeric insert of AV, AVI, and AVII. The dimeric insert was significantly more effective at directing packaging than the monomeric insert, and packaging of the monomer was favored in the forward orientation relative to the reverse orientation. Importantly, the insertion of a forward dimer resulted in full restoration of packaging efficiency to wild-type levels. These data suggest that a dimer of AV, AVI, and AVII can function as a minimally sized, yet maximally functional, packaging domain in an orientation independent manner.

We next introduced (AV-VII)^2 into an even larger deletion background, eliminating sequences between nt 53 and 814. The nonviable phenotype of the parent virus 53/814 was also rescued by insertion of (AV-VII)^2 in either orientation. Packaging efficiency was three- to fourfold less than that of the coinfecting wild-type virus and was independent of the orientation of the insert. Overall growth in a single infection was reduced 82-fold in the forward, and 16-fold in the reverse, orientation. Again, the plaque size with these mutants was small, which may partly explain the large difference between single and coinfection results.

Selection of revertants from a packaging-deficient adenovirus. To generate a packaging-deficient virus as a parent for the selection of revertants, we constructed a virus with a deletion of sequences between nt 194 to 458 which eliminated the viral packaging domain (Fig. 1A). Six copies of AVI (21-bp oligonucleotide [see Materials and Methods]) were introduced at the site of the deletion, as well as four E1A enhancer repeats (2, 4) to ensure efficient E1A transcription (Fig. 6A). The parent virus was plaque purified from 293 cells, and a number of independent isolates were passaged on A549 cells to minimize the chance of recombination events between adenovirus left-end sequences integrated in the genome of 293 cells and parental genomes. During the first growth cycle on 293 cells, the parent virus had already acquired two additional copies of AVI (Fig. 6A); we therefore refer to it as 21/8 E1A$^+$. After 12 passages on A549 cells, an accelerated onset of cytopathic effect was evident. Viruses were plaque purified and amplified, and growth and packaging efficiencies of several independent isolates were determined on 293 cells. Figure 6A shows the



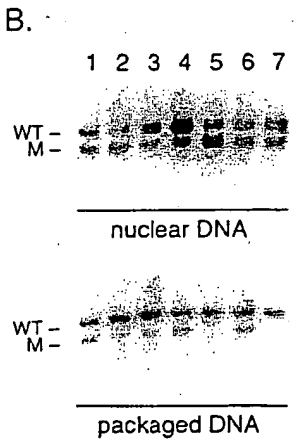


FIG. 3. Mutations surrounding AV and AVI. (A) Mutant viruses with substitution mutations and an insertion mutation in the region between AV and AVI, as well as substitution mutations to the left of AV and to the right of AVII. The nomenclature and the results from single infections and coinfections are as described for Fig. 2A. NV, mutant virus was nonviable in at least three transfection experiments. (B) Southern blot analysis of total nuclear and virion DNA isolated from 293 cells coinfected with wild-type (WT) virus and the mutant (M) viruses depicted in panel A (performed as described in the legend to Fig. 2C). The mutant viruses analyzed were LS 334–336 (lanes 1), LS 348–350 (lanes 2), LS 351–353 (lanes 3), LS 357–359 (lanes 4), LS 360–362 (lanes 5), and LS 376–378 (lanes 6).

results of single infections and coinfections of the parent virus and four revertants. The parent virus 21/8 E1A$^+$ was reduced 32-fold in growth in a single infection relative to the wild type. Packaging efficiency was reduced correspondingly in a coinfection with wild-type virus. Left-end sequences of the revertants were analyzed by restriction digestion and sequencing and were found to harbor precise amplifications of AVI, with 12, 16, 17, and 20 elements present, compared to 8 elements in the parent virus. All revertant viruses exhibited a significant (11- to 16-fold) increase of viral growth in the single infection and a comparable increase of packaging efficiency in the coinfection relative to the parent virus, indicating that amplification of AVI provided the viruses with a competitive advantage. The fact that revertants of 21/8 E1A$^+$ evolved through the amplification of A repeats presents genetic evidence that these sequences function as independent units in viral DNA packaging.

An example of a Southern blot prepared from 293 cells coinfected with wild-type virus and the parent virus (21/8 E1A$^+$) or its revertants is shown in Fig. 6B. The relative mobility of the left-end fragment varies between the parent virus and the revertants, depending on the number of copies of AVI present. Each of the revertants packaged viral DNA in the coinfection experiment with increased efficiency relative to the parent virus. The faint band above the AVI fragment with the parental virus (Fig. 6B, lane 1) presumably represents genomes with already multimerized packaging repeats, suggesting a strong evolutionary pressure for the presence of more than eight copies of AVI. We also tested the packaging abilities of the parent virus and of the revertant viruses carrying 12, 16, and 17 copies of AVI in a coinfection with the revertant car-

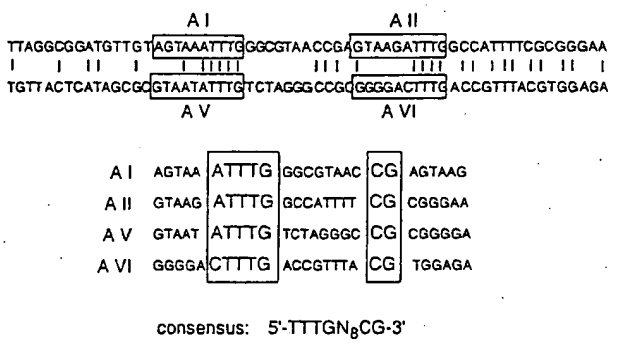


FIG. 4. Alignment of AI, AII, AV, and AVI. The packaging repeats are boxed; invariable nucleotides are indicated by lines between the sequences. Shown below is an alignment of the individual A repeats. Invariant nucleotides between these repeats are indicated by large letters. A new consensus motif for the Ad5 packaging elements is shown at the bottom.

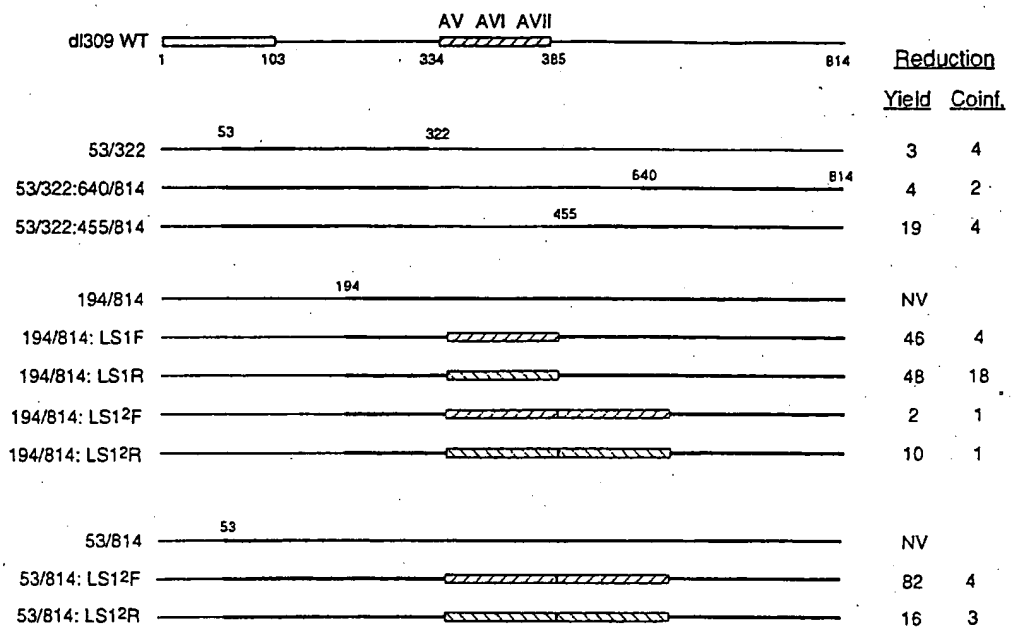


FIG. 5. Viruses with minimal packaging domains. A schematic representation of left-end sequences of *dl*309 wild-type (WT) virus is shown at the top. The ITR is represented by an open box; AV through AVII (nt 334 to 385) are represented by a hatched box. Numbers represent nucleotide positions relative to the left end. Deletions in individual viruses are indicated by a solid line; the last nucleotides remaining on either side of the deletion are indicated and are numbered relative to the left-end terminus. The names of the mutant viruses in the top set represent the deletions introduced into left-end sequences. The middle and bottom sets of mutant viruses contain deletions between nt 194 and 814 and nt 53 and 814, respectively. A monomer (AV-VII) or dimer $(AV\text{-}VII)^2$ of an oligonucleotide containing AV through AVII was inserted at the deletion site junction. The orientation of the AV-VII fragment is indicated as forward (F) or reverse (R). The results of single and coinfections of 293 cells are expressed as described in the legend to Fig. 2A.

rying 20 A repeats. These viruses differ from each other only by the copy number of AVI present in place of the viral packaging domain. Figure 6C shows Southern blots prepared from total nuclear DNA as well as packaged DNA from these coinfections. The uppermost band is shared by all viruses and is composed of the four E1A enhancer repeats in conjunction with the region between nt 458 and 1339. The lower fragments contain nt 1 to 194 as well as the AVI multimers. All viruses replicated to equal levels in a coinfection with the 21/20 $E1A^+$ virus (lanes 1 to 4). When packaged DNA was analyzed, however, packaging of the parent virus as well as the revertant virus with 12 A repeats was substantially reduced (lanes 5 and 6). Viruses harboring 16 and 17 copies of AVI packaged their DNA as efficiently as the 21/20 $E1A^+$ revertant (lanes 7 and 8). These data strongly support the notion that viruses with more packaging elements have a competitive advantage over viruses with fewer elements when they are challenged to compete with each other. All four revertants, when tested in a single infection, exhibited similar growth properties (Fig. 6A), which strengthens our hypothesis that *trans*-acting packaging components are required to bind the packaging repeats before DNA encapsidation can ensue. Such packaging factors may be limiting, which would result in preferential encapsidation of genomes containing more packaging elements in a coinfection experiment.

We also inserted 12 head-to-tail copies of AVI in either orientation into the 53/814 deletion as shown in Fig. 7. The resulting mutant viruses, 194/814:AVI/12F and 194/814:AVI/12R, were 22- and 48-fold reduced in the single infection, with a corresponding decrease in the coinfection of 17- and 21-fold. Sequencing of viral left-end fragments revealed more than 12 copies of AVI in both viruses, indicating that an amplification of packaging elements had taken place. At least 18 copies were present in mutant 194/814:AVI/12F, and at least 15 copies were present in mutant 194/814:AVI/12R. This amplification event is reminiscent of the multimerization of A repeats in revertants selected from a packaging-deficient mutant virus described above. These data confirm the results obtained with the selection of revertants carrying amplified packaging repeats.

DISCUSSION

Adenovirus DNA packaging is often likened to DNA packaging in double-stranded DNA bacteriophage systems such as phage lambda or ϕ29. Viral DNA is specifically selected from the pool of total DNA for its packaging, and insertion of the genome into preformed, empty capsids proceeds in a polar fashion. For these two phages, it has clearly been shown that phage-encoded packaging factors selectively bind packaging sequences located within the genomic left-end terminus. Formation of a nucleoprotein complex on the packaging domain then marks the respective molecule as a bone fide packaging substrate followed by recognition of the empty capsid and insertion of the DNA with left-to-right polarity (1, 9).

The identities of such packaging factors in the case of adenovirus are still unknown, but several lines of evidence strongly support the existence of limiting *trans*-acting packaging components. A number of packaging mutants were previously reported to exhibit a greater reduction in packaging efficiency in the coinfection experiment than was expected from single infections with the respective viruses. A model was proposed in which a competition for a limiting *trans*-acting packaging component would take place between mutant and wild-type vi-

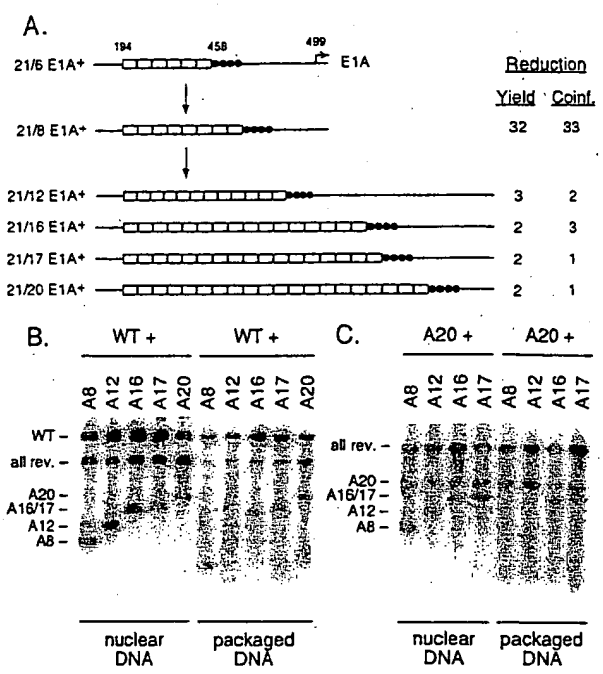


FIG. 6. Adenovirus packaging revertants. (A) Schematic representation of a packaging-deficient parent virus and the revertant viruses selected from it. The E1A transcriptional start site at nt 499 is represented by an arrow; E1A enhancer sequences are indicated by closed circles; open boxes represent a 21-bp oligonucleotide containing AVI (see Materials and Methods). The parent plasmid carried six AVI copies (21/6 E1A$^+$). The parental virus originally isolated had acquired two additional copies of AVI during the first round of growth (21/8 E1A$^+$). AVI was further amplified upon multiple passages of the 21/8 E1A$^+$ parent virus, as indicated by the names of the revertant viruses and the number of open boxes in the diagram. The data from single infections as well as coinfections of 293 cells are expressed as described in the legend to Fig. 2A. (B) Southern blot analysis of total nuclear and virion DNA isolated from 293 cells coinfected with wild-type virus and parent or revertant (rev.) viruses. Viral genomes were digested with *Xba*I and *Eco*RI and subjected to Southern blot analysis as described the legend to Fig. 2C. The wild-type (WT) *Xba* fragment is indicated on the left. The parent and revertant viruses carry an *Eco*RI site between the AVI multimers and the E1A enhancer repeats. Correspondingly, whereas the upper fragment is of equal length for all of these viruses, fragments representing the left-end terminus vary in mobility depending on the copy number of AVI present. These left-end fragments containing multiple copies of AVI are indicated on the left. The mutant viruses tested were the parent virus 21/8 (lanes 1) and the revertant viruses 21/12 E1A$^+$ (lanes 2), 21/16 E1A$^+$ (lanes 3), 21/17 E1A$^+$ (lanes 4), and 21/20 E1A$^+$ (lanes 5). (C) Southern blot analysis of total nuclear and virion DNA isolated from 293 cells coinfected with the revertant carrying 20 copies of AVI and its parent virus carrying eight A repeats or other revertants carrying 12, 16, and 17 copies. Southern blot analysis was performed as described in the legend to Fig. 6B. Fragments representing the left-end termini of the different viruses are indicated on the left.

ruses, with the wild-type packaging domain providing a competitive advantage (16). This model was strongly supported by a cotransfection experiment in which an excess of unlinked packaging-domain sequences dramatically inhibited viral growth without affecting DNA replication and late transcription, presumably by titrating packaging factors away from virus genomes (17). A third line of evidence is presented in this report and involves the evolution of packaging-competent viruses from a packaging-deficient parent virus through the amplification of packaging repeats (Fig. 6A). Twelve, 16, 17, and 20 copies of A repeats in place of the packaging domain resulted in an improvement of virus growth and packaging ability to wild-type or near-wild-type levels. When challenged in a coinfection experiment, viruses carrying more A repeats displayed a competitive advantage over viruses with fewer repeats (Fig. 6C). Specifically, 12 copies of AVI did not allow significant levels of packaging in a coinfection with a virus containing 20 A repeats, whereas 16 and 17 copies did. It appears likely that this is the result of a competition for the binding of a limiting packaging component in which more packaging elements increase the likelihood of binding of such a component.

Our results provide genetic evidence that packaging elements, in this case AVI, act as independent functional units in viral DNA packaging. To ensure that the reverted phenotype is a direct consequence of the presence of additional copies of A repeats and not due to unidentified second-site mutations, we reconstructed a recombinant adenovirus carrying 12 copies of AVI inserted into a deletion between nt 194 and 458. Left-end sequences of this recombinant virus including the packaging domain are identical to the 21/12 E1A$^+$ revertant left end. As predicted, in a single infection this recombinant virus grew to the same level as the revertants carrying 12 or more A repeats (data not shown). This result argues against the possibility that revertants carried other second-site mutations that augmented packaging efficiency. An alternative explanation for the stimulation of DNA packaging through the amplification of packaging repeats is an attempt to optimize the spacing between the packaging elements and the left-end terminus of the adenovirus genome. This possibility appears unlikely for two reasons. First, a DNA fragment containing AV, AVI, and AVII functioned efficiently for packaging as a dimer when positioned at nt 53 (Fig. 5). Amplification of this segment was not observed, and so packaging elements can function when located near the terminus of the viral genome. Second, amplification of A repeats was observed when multimers of AVI were located at nt 53 or 194 (Fig. 6 and 7). The fact that selective pressure for the multimerization of A repeats exists in two situations in which spacing between the packaging domain and the left-end terminus is substantially different argues against a selection to make specific spatial changes and for a selection for the pres-

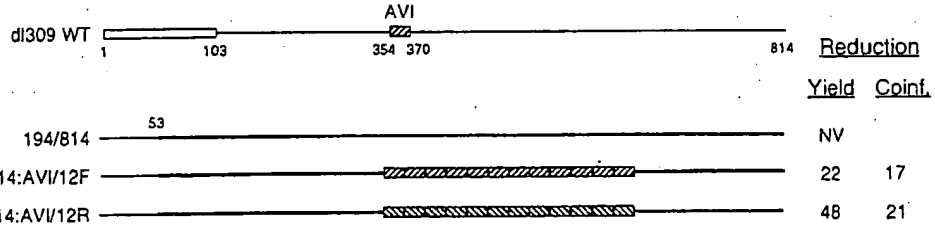


FIG. 7. AVI specifies packaging. A schematic representation of left-end sequences of *dl*309 wild-type virus is shown at the top. The ITR is represented by an open box; AVI (nt 354 to 370) is represented by a hatched box. Numbers represent nucleotide positions relative to the left end. The mutant viruses contain a deletion between nt 53 and 814 and the insertion of 12 copies of AVI with mutants 194/814:AVI/12 in the forward (F) or reverse (R) orientation. The results of single infections and coinfections of 293 cells are expressed as described in the legend to Fig. 2A.

ence of more packaging elements. The two possibilities, however, are not mutually exclusive.

In addition to a packaging factor(s) presumed to bind the A repeats, factors bound to ITR sequences also have been hypothesized to play a role in viral DNA packaging. The adenovirus packaging domain, although positionally flexible to a certain extent, has to be located near the ITR to maintain activity (20). Two mutant viruses define the minimal ITR sequences that may be involved in DNA packaging [Fig. 5, 53/814:(AV-VII)^{2}F and 53/814:(AV-VII)^{2}R]. These viruses carry packaging domains in the context of the 53/814 deletion and display near-wild-type packaging efficiency. Only the left-most 53 nt, the core replication sequences, are present to the left of the packaging domain with these mutant viruses. Therefore, if factors bound to the left-end terminus do function in viral DNA packaging, they would have to serve a dual function in DNA replication as well as encapsidation of the genome. In support of the possible involvement of replication factors in adenovirus DNA packaging, certain mutations in the adenovirus terminal protein (TP), which is covalently attached to the 5′ terminus of the viral genome, inhibit virus growth in vivo but do not diminish terminal protein replication function in vitro (14, 15, 28). Such a result is consistent with a dual role for TP in replication and viral DNA packaging. Interestingly, in the case of bacteriophage ϕ29, which especially resembles adenovirus by way of a protein-primed DNA replication mechanism and the polar encapsidation of a nonconcatemeric genome, the TP primer for DNA replication, gp3, is also an enhancer of DNA packaging (1).

We attempted to define minimal packaging sequences with maximal packaging activity in vivo. A dimer of a fragment containing AV, AVI, and AVII rescued packaging efficiency in a coinfection experiment to wild-type levels in the 194/811 deletion mutant background and to near-wild-type levels in the 53/811 mutant background (Fig. 5). Therefore, this fragment constitutes the minimal packaging domain so far defined which is necessary and sufficient for viral DNA packaging in vivo. The AV-VII dimer rescued viral packaging significantly better than a multimer of AVI in the 53/811 mutant background (compare Fig. 5 and 7). Previous mutational studies identified AVI as the functionally most important packaging element in the context of a virus lacking AI through AIII (17). The fact that multimers of AVI could not reconstitute viral packaging to the extent that a combination of AV, AVI, and AVII did, even with a higher copy number of repeats present, suggests that a combination of different A repeats supports viral packaging better than only one type of element. This in turn could implicate more than one protein component to be part of the viral packaging machinery. To achieve maximal packaging efficiency, distinct factors would be required to bind different packaging elements followed by recognition of the prohead and subsequent encapsidation. The definition of minimal packaging domains with maximal packaging activity in vivo may prove useful for the construction of new helper viruses in the field of gene therapy to minimize target sequences for homologous recombination between gene therapy and helper virus as well as to maximize insert size.

It was previously established that adenovirus packaging repeats are functionally redundant (16, 17). Despite this redundancy, individual elements are not functionally equivalent but follow a hierarchy of importance. Early alignments identified a loosely conserved consensus motif, GTN$_{3-4}$TTTG, for the packaging elements. These findings were based on extensive analyses of viruses carrying deletion mutations as well as linker scanning mutations overlapping individual packaging elements in the context of a minimal packaging domain (16, 17). AI and

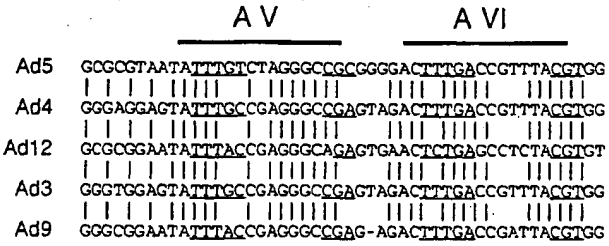


FIG. 8. Alignment of putative packaging repeats in different adenovirus subgroups. The nucleotide sequences corresponding to AV and AVI are shown for Ad5 (subgroup C), Ad4 (subgroup E), Ad12 (subgroup A), Ad3 (subgroup B), and Ad9 (subgroup D). The positions of AV and AVI in Ad5 are shown by lines above the sequences. Nucleotides identical between all subgroups are indicated by vertical lines.

AII, as well as AV and AVI, are the functionally dominant repeats, and they are also separated from each other by 21 bp. Factors bound to these repeats would be located on the same side of the DNA double helix to potentially interact with each other and/or with factors bound to the leftmost 53 nt of the adenovirus genome to allow for DNA encapsidation. In studies to address the importance of 21-bp spacing between AI and AII, as well as AV and AVI (Fig. 2 and 3), we identified a CG dinucleotide, located in the identical position relative to AI, AII, AV, and AVI, that is 11 bp downstream of the middle thymidine residue within the thymidine triplet (Fig. 4). Our mutational studies suggest that the 11-bp spacing between the TTTG and the downstream CG is important but that the 21-bp spacing between different packaging elements is not required, at least not with respect to AI and AII (Fig. 2B). An alignment of the four functionally dominant packaging repeats together with our mutational studies defines a more extended consensus motif, 5′-TTTGN$_8$CG-3′ (Fig. 4). Interestingly, these sequences also are conserved between different adenovirus subgroups in the regions corresponding to AV and AVI (Fig. 8). In vivo, the presence of the CG dinucleotide was found to be critical downstream of AI, AII, and AV but not downstream of AVI (Fig. 2 and 3). It is possible that functional redundancy applies not only to the AT-rich part of the new consensus motif but also to the CG sequence.

A single *trans*-acting packaging factor could interact with both conserved parts of the consensus motif, and it is noteworthy that the 11-bp spacing between the TTTG and CG sequences would present both halves of the consensus sequence on the same face of the DNA helix. Alternatively, the bipartite nature of the consensus motif could imply that distinct factors bind to the two conserved regions. Since different individual packaging repeats or combinations of repeats do not appear to be functionally equivalent, different packaging factors may interact with different individual A repeats. If one or more host cell factors are involved in adenovirus packaging, this may reflect the ability to recruit different binding proteins depending on the cell type infected and the predominance of the respective cellular factors in that cell type. Consequently, functional redundancy of the packaging elements could constitute one of many strategies that the virus has developed to allow for its well-documented ability to infect a wide variety of cell types.

ACKNOWLEDGMENTS

We thank our colleagues for many helpful discussions and Tina Philipsberg for excellent technical help.

This research was supported by funding from the Cystic Fibrosis Foundation (grant CFF Z161) and Public Health Service grant CA28146 from the National Cancer Institute to P.H.

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1. REAL PARTY IN INTEREST

The real party in interest in the present appeal is:

Merck and Co., Inc.

One Merck Drive

Whitehouse Station, New Jersey 08889

having acquired the entire right, title and interest for U.S. Patent Application Serial No. 09/890,836 from the Applicants, Andrew Bett, Volker Sandig and Rima Youil, by way of an Assignment.

2. RELATED APPEALS AND INTERFERENCES

No related appeals or interferences are known to appellants or appellants' legal representative which will directly affect or be directly affected by or have bearing on the Board's decision in this appeal.

3. STATUS OF CLAIMS

Claims 1-15 are presently pending in the application. Claims 1-15 stand finally rejected under 35 U.S.C. §112, second paragraph. Claims 1-5 and 7 and 6, 8 and 9-12 stand finally rejected under 35 U.S.C. §102 (b). The rejections of claims 1-15 under §112 and claims 1-5 and 7 and 6, 8 and 9-12 under §102 (b) are being appealed.

4. STATUS OF AMENDMENTS

In an Office Action dated January 14, 2004, the Examiner finally rejected claims 1-15. In response thereto, Applicants subsequently filed an Amendment on April 14, 2004 canceling claim 8, amending claims 1, 6, 7 and 10, and maintaining claims 4 and 14 which had been previously presented. Applicants filed a Supplemental Amendment on May 25, 2004 amending claim 1. The Supplemental Amendment erroneously indicated that claim 10 had been currently amended when it should have been listed as one previously presented. The Supplemental Amendment was filed in conformance with Applicants' legal representative's understanding of the suggestions made by the Examiner during telephonic interviews conducted on May 20 and 21, 2004.

An Advisory Action, dated May 24, 2004, and an Interview Summary, dated May 25, 2004, respectively, were received on May 27, 2004, subsequent to the filing of the Supplemental Amendment summarizing the telephonic interviews conducted on May 20, 2004 and May 21, 2004, respectively. The Advisory Action indicated that the Amendment was not entered in the case.

The claims that were pending as of the Final Office Action are appended as Appendix I. The claims that were presented, but not entered, in the Supplemental Amendment are appended as Appendix II.

For the convenience of the Board of Appeals and Interferences, three copies of the papers listed above are enclosed.

Also enclosed are three copies of the following publications discussed during the prosecution and in this Appeal Brief:

(a) Hardy *et al.*, International Patent Publication WO 97/32481 ("Hardy");

(b) Gräble, M. and Hearing, P., J. Virol., May 1990, Vol. 64, No. 5, p. 2047-2056 ("Gräble and Hearing, 1990");

(c) Gräble, M. and Hearing, P., J. Virol., Feb. 1992, Vol. 66, No. 2, p. 723-731 ("Gräble and Hearing, 1992"); and

(d) Schmid, S. and Hearing, P, J. Virol., May 1997, Vol. 71, No. 5, p. 3375-3384 ("Schmid and Hearing").

5. SUMMARY OF THE INVENTION

The embodiment of the invention that is under consideration in this application is directed to a novel element that has been incorporated into known helper virus vectors. The novel element is an adenovirus packaging signal cassette having low homology to, and less activity than, a corresponding wild-type packaging signal. The modified packaging signal is used in a helper virus to decrease recombination and generation of the virus.

A. Background

Homology between a helper and the helper-dependent adenoviral vector encourages recombination events between the two, resulting in unwanted changes in the structure of the helper-dependent adenoviral vector of the helper virus, and leading to an increased contamination by helper virus. Sequences for different low homology excisable packaging signal cassettes can be designed by one of ordinary skill in the art using the wild-type packaging signal sequences. The wild-type packaging signal of adenovirus serotype 5 is formed by at least seven functional units called A repeats, which are located between nt 230 and nt 380 of the genome. The A elements have the consensus sequence ATTTGN8CG, identified by Schmid and Hearing.

B. Invention

The low homology packaging signal cassette of the instant invention comprises a modified packaging sequence which fulfills the role of an adenovirus packaging signal and has low

homology relative to a corresponding wild-type adenovirus packaging signal. The modified packaging sequence of the instant invention has (1) fewer packaging elements, i.e. less A repeats, than the wild-type adenovirus packaging signal and (2) A elements that have been changed from their corresponding wild type. Specifically, in the claimed invention Applicants have found that a low homology packaging signal could be designed using less than seven A elements. In a preferred embodiment, the modified packaging signal has two to six A elements. The embodiment of the invention further comprises A elements that have been modified relative to the corresponding wild-type sequence in order to reduce contiguous sequence homology. In the modified A element, the eight ambiguous nucleotides (N8) of the consensus sequence within each A element have been replaced by sequences taken from a different A element. By way of example, the eight nucleotides with the first A element ("A1") were replaced by those from the fifth ("AV"), while the eight nucleotides with the second A element ("AII") with the nucleotides from the sixth A element ("AVI"). In addition, a modified A element can be created by changing the corresponding wild-type nucleotides to those of the consensus sequence, such as was done in the instant invention by creating a new A element between the AII and AIII starting 21 base pairs after AII. A modified A element can also be created by changing one or more of the unambiguous nucleotides of the consensus sequence such as was done in AIV; ATTTTGTGTT (SEQ ID NO. 2) was changed to ATTTTGTTGT (SEQ ID No. 3).

The following illustrates how the elements of claims 1-15 as they are presented in the Supplemental Amendment read on the specification.

Claim 1

Claim element	**Where found in the specification**
a nucleic acid molecule comprising a low homology packaging signal cassette	page 3, lines 19-22; page 5, lines 7-9
flanked by a recombinase recognition sequence	page 3, lines 24-27
a modified adenovirus packaging signal	page 3, lines 31-33; page 4, lines 1-7
having one to five A elements	page 11, lines 17-23; Example 2
each A element having a consensus sequence of ATTTGN8CG (SEQ ID NO. 1)	page 11, lines 15-17; Example 2
where N8 of each A element is replaced by the N8 sequence of a different A element	page 11, lines 25-34; Example 2
all N8 sequences are not identical	page 11, lines 25-34; Example 2

Claim 6

Claim element	Where found in the specification
maximum, 23 bp of contiguous sequence homology	page 4, lines 1-7
relative to a wild-type packaging signal	page 4, lines 1-7
at a portion of the sequence other than the A elements	page 4, lines 1-7; Example 2

Claim 7

Claim element	Where found in the specification
2-3 times less efficient than said wild-type signal	page 4, lines 8-15
relative to adenovirus within the same cell line	Page 4, lines 8-15

6. ISSSUES

There are three issues on appeal:

(A) whether claims 1-15 are indefinite under 35 U.S.C. §112, second paragraph;

(B) whether claims 1-5 and 7 are anticipated under 35 U.S.C. §102(b) by Hardy et al., WO 97/032481; and

(C) whether claims 6, 8 and 9-12 are anticipated under 35 U.S.C. §102(b) by Hardy et al., WO 97/032481.

7. GROUPING OF CLAIMS

With respect to the rejection under 35 U.S.C. §112, second paragraph, all the claims stand or fall together.

With respect to the rejection under 35 U.S.C. §102 (a) all the claims stand or fall together.

With respect to the rejection under 35 U.S.C. §102 (b) all the claims stand or fall together.

8. ARGUMENT

A. Summary of the Examiner's Position

The Appellants believe that the Examiner's reason for holding that claims 1-15 are indefinite can be summarized as follows:

Since the claims are directed to a low homology packaging signal, the claims must contain a function or property of the packaging signal that explicitly confers low homology to the construct relative to a wild-type signal.

Since claim 7 is directed to a modified packaging signal that is 2-3 times less efficient it must be definite as to how this efficiency is to be measured relative to a wild-type signal.

Since claim 8 is directed to an A element, it must be definite as to what is the consensus sequence.

Since claim 10 is directed to a helper virus it must be definite as to how the virus can be the nucleic acid of claim 6.

The Appellants believe that the Examiner's reasons for holding that claims 1-5 and 7 and 6, 8 and 9-12 are anticipated can be summarized as follows:

Hardy *et al.*, WO 97/32481 ("Hardy") disclosed the use of a low homology packaging signal containing A elements. Applicants use of the term "low homology" is insufficient to distinguish the claimed construct from that disclosed by Hardy. Applicants use of additional features in dependent claims is insufficient to distinguish the claimed invention from Hardy in that the claim from which they depend from is not novel.

A. Summary of the Appellants Position

With respect to the claims as they appear in the Supplemental Amendment and the indefiniteness rejection:

Applicants have amended claim 1 to incorporate the structure of previous claim 8 to further define the low homology packaging signal as suggested by the Examiner in a telephone interview. Applicants have amended claim 1 to further define the modified packaging signal as having one to five A elements, each A element having a consensus sequence (ATTTGN8CG, SEQ ID NO. 1), with N8 being replaced by the N8 sequence of a different A element and all A elements are not identical.

Applicants have amended claim 7 to further define and distinguish the claimed invention by specifying the location of the 23 bp of contiguous sequence homology.

Applicants have amended claim 10 to further define and distinguish the claimed invention by specifying that a helper virus comprises the nucleic acid of claim 6.

With respect to the claims as they appear in the Supplemental Amendment and the anticipation rejections:

Hardy et al. does not teach all of the elements of the modified packaging signal that comprises the low homology packaging signal claimed by the Applicants. Hardy does not teach any A element having a modified consensus sequence. Hardy does not teach the use of 23 bp of contiguous sequence homology at a portion of the sequence other than the A elements. Hardy does not teach a packaging signal that is less efficient relative to an adenovirus within the same cell line.

Claims 1-15 are patentable under 35 U.S.C. §112, second paragraph, as they clearly convey and distinctly claim the subject matter claimed therein.

Claims 1-15 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. In particular, the above claims were alleged to be indefinite as to the metes and bounds of what a "low homology" packaging signal is relative to a wild-type signal. Claim 7 was rejected as it was alleged that it was not clear to what the phrase "2-3 times less efficient" relates. Claim 8 was rejected for alleged lack of clarity as to what is the consensus sequence. Claim 10 was rejected for alleged lack of clarity as to how a nucleic acid can be a helper virus. Applicants respectfully traverse these rejections and submit that they are not sufficient.

In the Supplemental Response, Applicants have amended claim 1 to more distinctly define and distinguish the invention by adding the feature that the packaging signal has one to five A elements and that each A element has a consensus sequence, ATTTGN8CG (SEQ ID NO. 1), which features were previously in dependent claim 8. Claim 1 has been further amended to specify that the ambiguous N8 sequence of each A element is replaced by the N8 sequence of a different A element and that all N8 sequences are not identical.

Similarly, Applicants have amended claim 7 to further recite that the decreased efficiency of the modified packaging signal of claim 5 (which depends ultimately from claim 1) relative to a wild-type packaging signal is relative to an adenovirus within the same cell line. While Hardy may suggest that constructs in different host cells have different packaging (page 39, line 18-20), a full reading of that paragraph makes it evident that the comparison the reference is making is across cell lines (page 39, lines 13-20). Applicants have defined "less efficient" on page 4, lines 8-15 of the Specification, and have

defined that the claimed term refers to a comparison of the modified packaging signal relative to a wild-type adenovirus packaging signal within the same cell line, not across different types of cell lines.

The embodiment of the invention set forth in claim 8 has been incorporated into claim 1and claim 8 has been canceled, rendering this rejection moot.

Applicants have amended claim 10 in the Supplemental Response to clarify that the claimed helper virus comprises the nucleic acid molecule of claim 6.

Applicants assert that, based on the amendments made and the information provided within the Specification, one of ordinary skill in the art would be able to appreciate the nature of the low homology, modified adenovirus packaging signal claimed and to further find and identify the species encompassed. This alone supports the mandates of 35 U.S.C. §112 which requires only that the scope of the claim be "clear to a hypothetical person possessing the ordinary level of skill in the pertinent art," see MPEP §2171.

In evaluating claims with regard to Section 112, "definiteness of claim language must be analyzed, not in a vacuum, but in light of : (a) the content of the particular application disclosure; (b) the teachings of the prior art; and (c) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made," see MPEP §2173.02. In this regard, Applicants point out that the Specification, the application disclosures which details, *inter alia*, all of the structural and functional characteristics discussed above, describes the A elements and the changes made to the A elements in detail more than sufficient to enable "one possessing the ordinary level of skill in the pertinent art" to ascertain the scope of the patent claims.

The claim terminology "low homology" as used relevant to a modified adenovirus packaging signal is not a means for rejecting the claims, as the term itself is very specific when used in the context of adenovirus packaging signals. Proper enablement does not equate with importing all characteristics of the packaging signal detailed in the Specification into the claims. As indicated above, "whether a claim is invalid for indefiniteness requires a determination whether those skilled in the art would understand what is claimed when the claim is read in light of the specification," Morton International, Inc. v. Cardinal Chemical Co., 28 USPQ 2d 1190, 1194 (Fed. Cir. 1993), on remand from, 26 USPQ 2d 1721 (1993).

The claimed packaging signal molecules are thoroughly described and detailed in the Specification and the terminology used is certainly sufficient to put one of ordinary skill in the art on notice of the scope of the claims. Further, the claims incorporate sufficiently descriptive elements with antecedent support in the Specification in order to clearly and distinctly convey exactly what the invention entails.

Applicants, therefore, submit that the instant claims are not indefinite, but rather sufficiently descriptive to very clearly and distinctly convey the scope of the invention.

Applicants, therefore, respectfully request that this objection be overturned.

Claims 1-5 and 7 are not anticipated by Hardy.

The above claims are rejected based on Hardy which discloses methods for producing, *in vivo*, helper-free, totally defective adenovirus vectors for use in gene therapy. The recombinant adenovirus vectors described therein are packaged using a helper virus which can be excised *in vivo* by recombination mediated by a recombinase. It has been asserted that Hardy teaches a packaging signal construct having low homology, i.e. a nucleic acid molecule for use as a helper virus that is inefficiently packaged (as taught on page 39). Applicants submit that in light of the claim amendments presented in the Supplemental Response, this is not a sufficient rejection.

As noted above, in the Supplemental Response Applicants amended claim 1, and in turn the claims depended therefrom, to include additional features, namely, the number and type of A elements. More specifically, claim 1 states that the modified packaging signal has one to five A elements, which is less than the seven A elements of the wild-type packaging signal, and wherein each A element has a consensus sequence, ATTTGN8CG (SEQ ID NO. 1). Further, it has been specified that the ambiguous N8 sequence of each A element is replaced by the N8 sequence of a different A element, such that all N8 sequences differ from their corresponding wild-type A element and all are not identical.

Hardy defines that the packaging signal used therein contains an adenovirus packaging site *in cis* for packaging of the DNA into the adenovirus vectors (page 16, lines 1-2). The packaging sites exemplified in Hardy are directed to mutations of the naturally occurring adenovirus packaging sites. Hardy states that, in addition to the naturally occurring adenovirus packaging sites, certain other DNA sequences have been shown empirically to function as packaging sites, i.e. synthetic packaging sites. Id. at lines 4-9. The only example given in Hardy of such a synthetic packaging site *in vivo* is given as a cross reference to Gräble and Hearing, 1990, in which one synthetic packaging site composed of six tandemly repeated copies of the A repeat was used as a packaging site. Id. at lines 15-19. A further review of the A repeats of Gräble and Hearing, 1990, makes it apparent that they utilized an A repeat (A/T-AN-A/T-TTTG) with a consensus sequence that differed from that claimed by the Applicants.[1]

[1] This consensus sequence was later modified by Gräble and Hearing, 1992 ($GTN_{3-4}TTTG$), and was modified yet again in Schmid and Hearing to the presently accepted A element consensus sequence ($ATTTGN_8CG$).

Thus, one of ordinary skill in the art would find no teaching either explicitly or inherently of the modified A elements claimed by the Applicants.

The Examiner has suggested in the May 25, 2004 Communication, received after the filing by Applicants' legal representative of the Supplemental Response, that Applicants should not claim a construct comprising one or two A elements, presumably inferring from Gräble and Hearing, 1990, that such a construct would not be functional. However, because Gräble and Hearing, 1990 used identical sequences (which were not the consensus sequences of the instant invention) in tandem as A elements and did not use a modified packaging signal comprising non-identical modified A elements (with the altered N8 sequences) which were not in tandem, one of ordinary skill in the art would not infer the teachings as to functionality of these constructs to the instant invention. On the contrary, upon a further review of the third construct in both Fig. 3 and Fig. 7 one skilled in the art may well infer that a construct which has two A elements that are not in tandem may function as a packaging signal *in vivo*.

Applicants submit that, absent any disclosure in either Hardy or Gräble and Hearing, 1990 as to the use of a modified packaging signal comprising the modified A elements, there can be no anticipation of claim 1 and the claims that depend therefrom. A valid anticipation requires "disclosure in [a] single prior art reference of each element of [the] claim under consideration," W.L. Gore & Assoc., Inc. v. Garlock, Inc., 721 F.2d 1540, 1554, 220 USPQ 303, 313 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984).

Applicants, therefore, respectfully request that this rejection be overturned.

Claims 6, 8 and 9-12 are not anticipated by Hardy.

The above claims are rejected based on Hardy which discloses methods for producing, *in vivo*, helper-free, totally defective adenovirus vectors for use in gene therapy. As stated above, the recombinant adenovirus vectors described therein are packaged using a helper virus which can be excised *in vivo* by recombination mediated by a recombinase. It has been asserted that Hardy teaches a heterologous packaging signal construct that is equivalent but not identical and not co-linear as indicated by genome position. It is further asserted that these constructs can be plasmids and can contain deletion of E1 and be a helper virus. Applicants submit that in light of the claim amendments presented in the Supplemental Response, this is not a sufficient rejection.

As noted above, the Supplemental Response amended Claim 6 to further specify that the 23 bp of contiguous sequence homology is relative to a wild-type packaging signal at a portion of the sequence other than the A elements. Claim 6 depends ultimately from claim 1 and thus incorporates the structural features included thereof. Applicants do not refute the assertions (set forth in an earlier Office

APPENDIX I

1. (original) A nucleic acid molecule comprising a low homology packaging signal cassette flanked by a recombinase recognition sequence, wherein said packaging signal cassette comprises a modified adenovirus packaging signal, provided that said modified packaging signal has low homology to a wild-type adenovirus packaging signal.

2. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *loxP*.

3. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *frt*.

4. (previously presented) The nucleic acid of claim 1, wherein said modified packaging signal is less efficient than said wild-type packaging signal.

5. (original) The nucleic acid of claim 4, wherein said wild-type packaging signal is human adenovirus serotype 5 packaging signal.

6. (currently amended) The nucleic acid of claim[[s]] 5, wherein the modified packaging signal comprises at a maximum, 23 bp of contiguous sequence homology with said wild-type packaging signal.

7. (original) The nucleic acid of claim 5, wherein said modified packaging signal is about 2-3 times less efficient than said wild-type signal.

8. (currently amended) The nucleic acid of claim 6, wherein said modified packaging signal comprises two to six A elements, each A element having a consensus sequence of ATTTGN$_8$GC (SEQ ID NO: 1).

9. (original) The nucleic acid of claim 6, wherein said nucleic acid is a plasmid.

10. (currently amended) The nucleic acid of claim 6, wherein said nucleic acid [[is]] comprises a helper virus.

11. (original) The nucleic acid of claim 10, wherein said helper virus does not contain an E1 gene.

12. (original) The nucleic acid of claim 11, wherein said helper virus comprises an E3 region with an insert of about 2.9 kb.

13. (original) The nucleic acid of claim 12, wherein said insert does not contain a promoter sequence.

14. (currently amended) A nucleic acid of claim 13 comprising an adenovirus E3 gene having an insertion of at least about 2.7 kb, provided that said insertion does not contain a promoter sequence.

15. (original) The nucleic acid of claim 14, wherein said insertion is a human intron sequence.

16 - 41. (canceled)

APPENDIX II

1. (currently amended) A nucleic acid molecule comprising a low homology packaging signal cassette flanked by a recombinase recognition sequence, wherein said packaging signal cassette comprises a modified adenovirus packaging signal having one to five A elements, each A element having a consensus sequence of ATTTGN8CG (SEQ ID NO:1), and where N8 of each A element is replaced by the N8 sequence of a different A element and all N8 sequences are not identical.

2. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *loxP*.

3. (original) The nucleic acid of claim 1, wherein said recombinase recognition sequence is *frt*.

4. (previously presented) The nucleic acid of claim 1, wherein said modified packaging signal is less efficient than said wild-type packaging signal.

5. (original) The nucleic acid of claim 4, wherein said wild-type packaging signal is human adenovirus serotype 5 packaging signal.

6. (previously presented) The nucleic acid of claim 5, wherein the modified packaging signal comprises at a maximum 23 bp of contiguous sequence homology relative to a wild-type packaging signal at a portion of the sequence other than the A elements.

7. (previously presented) The nucleic acid of claim 5, wherein said modified packaging signal is about 2-3 times less efficient than said wild-type signal relative to adenovirus within the same cell line.

8. (cancelled)

9. (original) The nucleic acid of claim 6, wherein said nucleic acid is a plasmid.

10. (currently amended) A helper virus comprising the nucleic acid of claim 6.

11. (original) The nucleic acid of claim 10, wherein said helper virus does not contain an E1 gene.

12. (original) The nucleic acid of claim 11, wherein said helper virus comprises an E3 region with an insert of about 2.9 kb.

13. (previously presented) The nucleic acid of claim 12, wherein said insert does not contain a promoter sequence.

14. (original) A nucleic acid of claim 13 comprising an adenovirus E3 gene having an insertion of at least about 2.7 kb, provided that said insertion does not contain a promoter sequence.

15. (original) The nucleic acid of claim 14, wherein said insertion is a human intron sequence.

P&T OFFICE ACKNOWLEDGEMENT

ATTORNEY	DATE
J. E. Switzer	12/20/04
CASE NUMBER/	SERIAL NUMBER
20377YP	09/890,836

DATE FILED
August 3, 2001

APPLICANT
Bett et al.

EXPRESS MAIL NO.
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